UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUÍMICAS

TESIS DOCTORAL

Estudio del modo de acción de nuevos inmunomoduladores para el desarrollo de estrategias terapéuticas alternativas en infecciones recurrentes del tracto genitourinario y cáncer

Study of the model of action of novel immunomodulators for the development of alternative therapeutic strategies for recurrent genitourinary tract infections and cancer

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Leticia Martín de la Cruz

Director

Óscar Palomares Gracia

Madrid

© Leticia Martín de la Cruz, 2023

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS QUÍMICAS

TESIS DOCTORAL

Estudio del modo de acción de nuevos inmunomoduladores para el desarrollo de estrategias terapéuticas alternativas en infecciones recurrentes del tracto genitourinario y cáncer

Study of the mode of action of novel immunomodulators for the development of alternative therapeutic strategies for recurrent genitourinary tract infections and cancer

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Leticia Martín de la Cruz

DIRECTOR

Óscar Palomares Gracia

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS

Departamento de Bioquímica y Biología Molecular

TESIS DOCTORAL

Estudio del modo de acción de nuevos inmunomoduladores para el desarrollo de estrategias terapéuticas alternativas en infecciones recurrentes del tracto genitourinario y cáncer

Study of the mode of action of novel immunomodulators for the development of alternative therapeutic strategies for recurrent genitourinary tract infections and cancer

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

Presentada por

Leticia Martín de la Cruz

Director

Óscar Palomares Gracia

Madrid, 2023

A mis padres y mi hermano

A Joaquín

AGRADECIMIENTOS

“Hay quien deja huella y hay quien marca el camino”
Elvira Sastre

Esta Tesis Doctoral no podría empezar de otra manera si no es dando las gracias a todas aquellas personas que me han acompañado a lo largo de este camino y que, inevitablemente, han dejado su huella tanto en lo profesional como en lo personal.

En primer lugar, me gustaría agradecer a mi Director de Tesis y mentor la oportunidad de realizar este trabajo. Gracias Óscar por haber confiado en mí desde el principio, por tus consejos, tu apoyo, tu dedicación y tu cercanía. Por inspirarme, exigirme tanto e impulsarme a mejorar. He aprendido tanto de ti y lo sigo haciendo cada día. También me gustaría agradecer a mi tutora Nieves su rápida ayuda en toda la labor burocrática de la UCM. Gracias a todo el Departamento de Bioquímica y Biología Molecular de la UCM por permitirme y ayudarme a realizar esta Tesis.

También dar las gracias a nuestros compañeros de Inmunotek y del Clínico su inestimable colaboración y ayuda constante. Especialmente a Marcos, Carmen, Silvia Sánchez Ramón y Lidia. En particular, por lo que supone para esta Tesis, gracias José Luis por promover proyectos tan interesantes en los que he podido participar, por las discusiones tan enriquecedoras y todas las nuevas ideas en cada reunión.

Thanks to Dr. Mihai G. Netea for giving me the opportunity to spend three months in his research group in Nijmegen. It was a wonderful experience, which has enriched me both as a scientist and as a person. Thanks to all of the AIG lab team members for accepting me as one of their own. Thanks to Özlem, Ilayda and Büsra for their continuous support along the way. Special thanks to Marisol, Daniela and Brenda for their friendship and for making room for me (both inside and outside the lab). Especialmente, me gustaría dar las gracias a Jorge por recibirme con los brazos abiertos. Gracias por tu disposición a discutir extensamente sobre los experimentos y charlar sobre ciencia, Países Bajos o la vida. Ha sido un placer conocerte personalmente y aprender de ti.

Gracias a Alba y Cris, mis compañeras, amigas y hermanas en el laboratorio y en mi día a día. Hemos aprendido, viajado, reído y nos hemos entendido tanto, que solo tengo palabras de agradecimiento y afecto hacia vosotras. Gracias por haber sido mi punto de apoyo todos estos años, sin vosotras esta etapa hubiera sido mucho más difícil. Gracias Mario y Carmen por la alegría que contagiáis siempre y por toda vuestra ayuda. Os merecéis todo lo bueno del mundo. Gracias a los que hoy están en este gran equipo del L19, Andrés, Ángel, Bego y Natalia. A las personas que me acogieron al principio en

el laboratorio, Sofía y Cris. Gracias también a los que han formado parte de él y han dejado huella, Jacobo y Bea.

A mis amigas de siempre, tan diferentes y tan unidas a la vez. Gracias por apoyarme en este camino, por preguntarme por los experimentos, por vuestra alegría cuando salen y por hacerme olvidar cuando no. Especialmente gracias a Marta, por haber entendido mis células y plasmarlas en esta bonita portada. A Juli y Ana por ser el mejor equipo, desde las prácticas de la carrera hasta hoy. Gracias por vuestra amistad para toda la vida.

Gracias a toda mi familia. Especialmente gracias a mis padres por su apoyo incondicional y por no cortarme las alas nunca. Gracias a los valores que me habéis inculcado me he convertido en la persona que soy ahora. Sobre todo, gracias por darme el mejor regalo, mi hermano. Gracias Enrique por ser mi compañero de aventuras y estar siempre presente. Eres una suerte. Gracias a mis queridos abuelos que sin comprender exactamente a lo que me dedicaba, han estado orgullosos de su nieta. Me siento muy afortunada de teneros a mi lado.

Por último, gracias Joaquín. Gracias por celebrar mis éxitos, incluso más que yo misma, y por el cariño y la paciencia cuando las cosas no salían bien. Por esto y por mil motivos más, quiero seguir recorriendo el camino contigo de la mano.

TABLE OF CONTENTS

ABBREVIATIONS 17

RESUMEN/ABSTRACT 27

INTRODUCTION 37

1. The immune system 39

1.1. An overview of the immune response and immunological memory 39

1.2. Dendritic cells as the link of innate and adaptive immune responses 41

1.2.1. Human myeloid dendritic cells (mDCs) 44

1.2.2. Human plasmacytoid dendritic cells (pDCs) 45

1.2.3. Human monocyte-derived dendritic cells (hmoDCs) 46

1.3. Mechanisms involved in polarization of naïve $CD4^+$ T cells 46

1.3.1. T helper 1 (Th1) cells 47

1.3.2. T helper 2 (Th2) cells 48

1.3.3. T helper 17 (Th17) cells 48

1.3.4. T helper 9 (Th9), 22 (Th22) and follicular helper (T_{FH}) cells 49

1.3.5. Regulatory T (Treg) cells 49

2. Immunometabolism 50

2.1. An overview of the main metabolic pathways in the immune system 50

2.2. Metabolic control of dendritic cell function 52

2.3. Metabolic pathways in trained immunity 53

3. Epigenetics 54

3.1. An overview of the main epigenetic modifications 54

3.2. Epigenetic modifications in trained immunity 55

4. Recurrent infections: genitourinary tract infections 56

4.1. Urinary tract infections (UTIs) 56

4.2. Vulvovaginal candidiasis (VVCs) 57

4.3. Trained immunity-based vaccines (TIbV) as alternative therapeutic approaches 58
4.3.1. Mucosal vaccines 59
4.3.2. Bacterial vaccine for RUTIs: MV140 60
4.3.3. Fungal vaccine for RVVCs: V132 62
5. Cancer 63
5.1. A brief overview of the cancer pathology 63
5.2. Immune system in cancer 65
5.2.1. Treg cells in cancer immunology 67
5.3. Tumor-associated carbohydrate antigens 69
5.4. Carbohydrate A10 (Ca10) 69
OBJECTIVES 73
MATERIALS AND METHODS 79
1. Material, media and reagents 81
2. Polybacterial and fungal preparations 81
3. Carbohydrate A10 (Ca10) 81
4. Isolation of different human cell subsets 82
4.1. Isolation of peripheral blood mononuclear cells 82
4.2. Purification of monocytes 82
4.3. Generation of human monocytes-derived DCs (hmoDCs) 83
4.4. Purification of naïve $CD4^+$ T cells 83
4.5. Purification of total blood DCs 83
4.6. Isolation of PBMC and monocytes for trained immunity experiments 84
5. Cell culture procedures 84
5.1. Culture of human DCs 84
5.2. Coculture experiments with naïve $CD4^+$ T cells 85

5.3. Regulatory T cell suppression assays 86
5.4. Trained immunity assays 87
6. Flow cytometry 87
6.1. Surface staining 87
6.2. Intracellular staining 88
6.3. Intranuclear staining 89
6.4. CFSE staining 89
7. Cytokine quantification by ELISA 89
8. Western blot 90
9. RNA extraction and complementary DNA synthesis 91
10. Real-time quantitative PCR 91
11. Metabolic studies 92
11.1. Metabolic studies in DCs 92
11.2. Metabolic studies in trained PBMC and monocytes 93
12. Chromatin immunoprecipitation (ChIP) assays 93
13. Animal models 94
13.1. Sublingual immunization mouse model with MV140/V132, MV140, V132 or control excipients 94
13.2. V132 immunization for trained immunity mouse model 94
13.3. *In vivo* Ehrlich tumor growth 95
13.4. Plasma collection and ELISA of induced serum-specific Ig 95
13.5. Spleen processing 95
13.5.1. Splenocyte culture for proliferation assays 96
13.5.2. Splenocyte culture for cytokine production 96
13.5.3. Splenocytes for Treg cell assays 96
13.6. Peritoneal lavage and peritoneal cell culture 96

13.7. Ca10 measurement by ELISA 96

14. Statistical analysis 97

RESULTS AND DISCUSSION 99

Block A: Study of the molecular mechanisms involved in the mode of action of fungal vaccine V132 and polybacterial preparation MV140 for recurrent genitourinary tract infections 101

Objective 1. Study of the molecular mechanisms involved in the capacity of MV140/V132 to immunomodulate human DCs 103

1.1. To study the capacity of MV140/V132 to regulate the phenotype and function of human DCs 103

1.2. To study the polarization of T cell responses induced by human DCs stimulated with MV140/V132 107

1.3. To study the main immune signalling pathways involved in the immunomodulatory effects of MV140/V132 in human DCs 110

1.4. To study metabolic and epigenetic changes induced by MV140/V132 in human DCs 112

1.5. To study the immunomodulatory properties of MV140/V132 in an *in vivo* mice model 115

Objective 2. Assess the capacity of V132, MV140 and MV140/V132 to induce trained immunity in preclinical human and mice models and elucidation of underlying molecular mechanisms 121

2.1. To study the capacity of V132, MV140 and MV140/V132 to induce trained immunity in human PBMC 121

2.2. To study the main metabolic and epigenetic changes promoted by V132 in human PBMC 123

2.3. To study the capacity of V132, MV140 and MV140/V132 to induce trained immunity in human monocytes 125

2.4. To study the capacity of V132 to induce trained immunity in an *in vivo* mice model 129

Block B: Study of the molecular mechanisms by which the tumoral carbohydrate Ca10 immunomodulates human DCs 135

Objective 3. Study of the capacity of the carbohydrate Ca10 to induce tolerogenic human DCs and the elucidation of underlying molecular mechanisms 137

3.1. To assess the potential relationship between circulating Ca10, tumor size and Treg cell generation in a solid Ehrlich tumor-bearing mice model 137

3.2. To study the capacity of the tumoral carbohydrate Ca10 to regulate the phenotype and function of human DCs 141

3.3. To study the main immune signalling pathways involved in the immunomodulatory effects of the carbohydrate Ca10 in human DCs 143

3.4. To study the metabolic changes and their contribution to the tolerogenic properties exerted by Ca10 in human DCs 145

3.5. To study the capacity of the tumoral carbohydrate Ca10 to generate Treg cells and the underlying molecular mechanisms involved in such effects 148

3.6. To assess the specific contribution of the carbohydrate moiety of Ca10 and the epitope A10 to condition the capacity of DCs to polarize T cell responses, with special focus on the generation of Treg cells 151

Objective 4. Study of the capacity of the tumoral carbohydrate Ca10 to reprogram monocyte differentiation into tolerogenic DCs 155

4.1. To study whether the tumoral carbohydrate Ca10 could reprogram human monocytes differentiation into tolerogenic DCs 155

4.2. To study the capacity of hmoDCs differentiated in the presence of Ca10 to polarize Treg cells 157

GLOBAL DISCUSSION 161

CONCLUSIONS 171

BIBLIOGRAPHY 177

APPENDIX 205

ABBREVIATIONS

1-MT: 1-methyl tryptophan

2-DG: 2-Deoxy-D-glucose

A10: monoclonal antibody A10

AA: antimycin A

Acetyl-CoA: acetyl coenzyme A

ADCC: antibody-dependent cell-mediated cytotoxic

AHR: aryl hydrocarbon receptor

Akt: protein kinase B

APC: allophycocyanin

APCs: antigen-presenting cells

AS DCs: AXL^+ $Siglec6^+$ DCs

ATP: adenosine triphosphate

AU: arbitrary units

BALT: bronchus-associated lymphoid tissue

BCG: *Bacille Calmette-Guérin*

Bcl6: B-cell lymphoma 6

BDCA: blood dendritic cell antigen

Breg: regulatory B

BSA: bovine serum albumin

Ca10 OX: oxidized Ca10

Ca10/hmoDCs: hmoDCs differentiated in the presence of Ca10

Ca10: carbohydrate A10

CAFs: cancer-associated fibroblasts

cAMP: cyclic adenosine monophosphate

CCR7: C-C chemokine receptor 7

cDCs: conventional DCs

CEA: carcinoembryonic antigen

CFSE: carboxy fluorescein succinimidyl ester

ChIP: chromatin immunoprecipitation

CLRs: C-type lectin receptors

CTLA4: cytotoxic T lymphocyte antigen 4

CTLs: $CD8^+$ cytotoxic T lymphocytes

Ctrl: control

CXCL: C-X-C chemokine ligand

CXCR5: C-X-C chemokine receptor type 5

DAMPs: damage-associated molecular patterns

DCIR: DC immunoreceptor

DCs: dendritic cells

DC-SIGN: DC-specific intercellular adhesion molecule 3-grabbing non-integrin

ECAR: extracellular acidification rate

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EF1a: elongation factor 1a

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular-signal regulated kinase

ET: Ehrlich tumor

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

FcRγ: Fc receptor γ-chain

FITC: fluorescein isothiocyanate

FLT3L: FMS-like tyrosine kinase ligand 3

FOXP3: Forkhead box P3

FTU: Formazine Turbidity Units

GAGs: glycosaminoglycans

GALT: gut-associated lymphoid tissue

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GATA3: GATA binding protein 3

GlcA: glucuronic acid

GlcNAc: N-acetylglycosamine

glycoPER: glycolytic proton efflux rate

GM-CSF: granulocyte-macrophage colony-stimulating factor

GMDPs: granulocyte-macrophage DC progenitors

GUTIs: genitourinary tract infections

H_2O_2: hydrogen peroxide

H3K27ac: histone 3 acetylation of lysine 27

H3K27me3: histone 3 trimethylation of lysine 27

H3K4me1: histone 3 methylation of lysine 4

H3K4me3: histone 3 trimethylation of lysine 4

H3K9me3: histone 3 trimethylation of lysine 9

HIF-1α: hypoxia inducible factor-1α

hmoDCs: human monocyte-derived DCs

HPSE treat.: Ca10 treated with Heparinases

HR2: histamine receptor 2

HRP: horseradish peroxidase

IC: isotype control

ICOS: inducible T-cell costimulator

ICOSL: inducible costimulator ligand

IDO: indoleamine 2,3-dioxygenase

IFN: interferon

IFNAR: type I interferon receptor

Ig: immunoglobulin

IKKα/β: IκB kinase complex

IL: interleukin

IL-10R: IL-10 receptor

IL-12R: IL-12 receptor

IL-1RA: interleukin-1 receptor antagonist

ILCs: innate lymphoid cells

ITAM: immunoreceptor tyrosine-based activating motif

ITIM: immunoreceptor tyrosine-based inhibitory motif

IκBα: inhibitor of NF-κB

JNK: c-Jun N-terminal kinase

Kyn: kynurenine

LAG3: lymphocyte activation gene 3

LDH: lactate dehydrogenase

LPS: lipopolysaccharide

mAbs: monoclonal antibodies

MALT: mucosa-associated lymphoid tissues

MAPKs: mitogen-activated protein kinases

mDCs: myeloid DCs

MDSCs: myeloid-derived suppressor cells

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

MTA: 5′-deoxy-5′-methylthioadenosine

mTOR: mammalian target of rapamycin

MyD88: myeloid differentiation primary response 88

$NaIO_4$: sodium metaperiodate

NALT: nasopharynx-associated lymphoid tissue

NF-κB: nuclear factor κB

NK: natural killer

NLRs: NOD-like receptors

NMR: nuclear magnetic resonance

NO: nitric oxide

NRP1: neuropilin 1

OCR: oxygen consumption rate

OD: optical density

Olig: Oligomycin

OPD: o-phenylenediamine dihydrochloride

OXPHOS: oxidative phosphorylation

p70S6K: p70S6 kinase

PAMPs: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cells

PD-1: programmed cell death-1

pDCs: plasmacytoid DCs

PD-L1: programmed death-ligand 1

PE: phycoerythrin

PerCP: peridinin-chlorophyll-protein

PGE2: prostaglandine E2

PHA: phytohaemagglutinin

PPP: pentose phosphate pathway

pre-cDC: conventional DC precursor

pre-pDC: plasmaytoid DC precursor

PRRs: pattern recognition receptors

PSA: prostate specific antigen

pTreg: peripherally-induced regulatory T

RALDH: retinaldehyde dehydrogenase

RLRs: RIG-like receptors

RORγt: retinoic acid-related orphan receptor γt

ROS: reactive oxygen species

Rot: rotenone

RUTIs: recurrent urinary tract infections

RVVCs: recurrent vulvovaginal candidiasis

SHP1: SH2-domain-containing protein tyrosine phosphatase 1

SOCS: suppressor of cytokine signalling

sTn: sialyl-Tn antigen

Syk: spleen tyrosine kinase

TAAs: tumor-associated antigens

TACAs: tumor-associated carbohydrate antigens

TAMs: tumor-associated macrophages

TANs: tumor-associated neutrophils

T-bet: T-box transcription factor

TCA: tricarboxylic acid

TCR: T-cell receptor

T_{FH}: T follicular helper

T_{FR}: T follicular regulatory

TGF-β: transforming growth factor β

Th: T helper

TIbV: trained immunity-based vaccines

TIGIT: T cell immunoreceptor with immunoglobulin and ITIM domains

TIM3: T cell immunoglobulin and mucin domain-containing 3

TLRs: Toll-like receptors

TME: tumor microenvironment

TNF: tumor necrosis factor

Treg: regulatory T

Trp: tryptophan

tTreg: thymus-derived regulatory T

UTIs: urinary tract infections

VEGF: vascular endothelial growth factor

VVCs: vulvovaginal candidiasis

WHO: World Health Organization

RESUMEN/ABSTRACT

RESUMEN

Las células dendríticas (DCs) son células presentadoras de antígenos profesionales que tienen un papel esencial en la inducción de respuestas inmunitarias adecuadas. Actúan como nexo de unión entre la inmunidad innata y adaptativa. Las DCs exhiben la capacidad dual de promover respuestas inmunitarias frente a patógenos potencialmente dañinos o de generar tolerancia frente a antígenos exógenos inocuos y antígenos propios. Este proceso se encuentra altamente regulado y depende de los antígenos y las señales que se encuentran en el ambiente. Alteraciones en la función de las DCs conducen al desarrollo de enfermedades inmunomediadas tales como infecciones recurrentes, cáncer, autoinmunidad o alergia.

Las infecciones y el cáncer constituyen un problema de salud muy importante con un gran impacto socio-económico a nivel mundial. Las infecciones del tracto genitourinario (GUTIs), que incluyen infecciones recurrentes del tracto urinario (RUTIs) y candidiasis vulvovaginales recurrentes (RVVCs), son enfermedades infecciosas bacterianas y fúngicas muy frecuentes. Actualmente, los tratamientos de referencia siguen siendo los antibióticos y antifúngicos, respectivamente. Sin embargo, el uso excesivo de estos fármacos conlleva un aumento de las resistencias microbianas y un efecto perjudicial en la microbiota. Las vacunas basadas en inmunidad entrenada (TIbV) son formulaciones capaces de inducir inmunidad entrenada, lo que confiere protección frente a los antígenos presentes en la vacuna y, además, frente a otros patógenos no contenidos en la preparación. Las TIbV se han propuesto como nuevas estrategias terapéuticas para las infecciones recurrentes. La vacuna sublingual MV140 es una preparación polibacteriana compuesta por bacterias enteras inactivadas por calor: 75% de Gram-negativas (25% *Escherichia coli*, 25% *Proteus vulgaris* y 25% *Klebsiella pneumoniae*) y 25% de Gram-positivas (*Enterococcus faecalis*). Además, ha mostrado eficacia clínica frente a RUTIs. MV140 activa DCs proporcionándoles la capacidad de generar células Th1, Th17 y células T productoras de IL-10. V132 es una preparación sublingual de *Candida albicans* inactivada por calor desarrollada para RVVCs. Debido a la asociación entre RUTIs y RVVCs, una nueva estrategia terapéutica que combine MV140 y V132 podría representar una alternativa adecuada para GUTIs. Sin embargo, se necesitan más estudios pormenorizados de los mecanismos moleculares subyacentes al modo de acción de esta combinación antes de su traslación a la clínica.

Durante el desarrollo tumoral, la transformación maligna de las células cancerígenas se encuentra asociada a cambios en los glicoconjugados de la superficie celular del tumor. Estos carbohidratos tumorales inhiben la respuesta inmunitaria frente

al tumor mediante diferentes mecanismos como puede ser la generación de células T reguladoras (Treg). El carbohidrato tumoral A10 (Ca10) es una estructura aberrante presente en la superficie celular del tumor de Ehrlich (ET) de ratón definido por la unión del anticuerpo monoclonal A10. Ca10 se libera espontáneamente del ET y es detectado en el suero de ratones portadores del tumor, pudiendo representar un mecanismo de escape tumoral. Sin embargo, se necesita una mayor investigación sobre las implicaciones de este carbohidrato Ca10 en la progresión tumoral.

Teniendo en cuenta todos estos aspectos, el objetivo global de esta Tesis Doctoral es avanzar en el conocimiento de los mecanismos moleculares mediante los cuales agentes exógenos de distinta naturaleza regulan la función de las DCs, incluyendo su capacidad de polarizar respuestas T. Por un lado, se van a estudiar los mecanismos moleculares implicados en el modo de acción de las vacunas de origen fúngico (V132) y bacteriano (MV140) empleadas para GUTIs. Además, se va a investigar la capacidad del carbohidrato tumoral Ca10 para inmunomodular DCs humanas.

En esta Tesis hemos demostrado que la combinación de MV140 y V132 en una única formulación actúa en DCs humanas promoviendo la generación de células T productoras de IFNγ e IL-17A, así como células Treg. MV140/V132 activa las vías de señalización de NF-κB, MAPKs y mTOR, induce un cambio metabólico hacia la glicólisis y producción de lactato, y la metilación de histonas en genes relacionados con la producción de citoquinas en DCs. Las células T $CD4^+$ del bazo de ratones inmunizados sublingualmente con MV140/V132 presentan una mayor proliferación tras la estimulación *in vitro* con los antígenos contenidos en la vacuna y con el antígeno no relacionado, fitohemaglutinina. Además, la inmunización sublingual de los ratones con MV140/V132 genera anticuerpos IgG e IgA específicos frente a los componentes incluidos en la vacuna. También hemos demostrado que V132 induce inmunidad entrenada en monocitos humanos, incrementando la producción de citoquinas pro-inflamatorias tras una estimulación secundaria con MV140. Los monocitos entrenados con V132 presentan un cambio metabólico caracterizado por una mayor glicólisis y fosforilación oxidativa, así como una reprogramación epigenética que favorece la transcripción de los genes pro-inflamatorios *IL6* y *TNFA*. La inmunización de ratones con V132 induce inmunidad entrenada en células peritoneales y esplenocitos, lo que contribuye a potenciar las respuestas IL-17 en ratones tratados con MV140 tras la estimulación *in vitro* con MV140.

En esta Tesis también hemos demostrado que el carbohidrato tumoral Ca10 favorece la generación de DCs humanas tolerogénicas con la habilidad de polarizar

células Treg funcionales con capacidad supresora. Los mecanismos implicados en este proceso dependen parcialmente de PD-L1, IL-10, IDO, la vía de mTOR y la glicólisis. También hemos demostrado que el proteoglicano de heparán sulfato presente en la estructura de carbohidrato del Ca10 es esencial para la inducción de DCs tolerogénicas y la polarización de células Treg. Nuestros resultados demuestran correlaciones positivas entre los niveles de Ca10 en suero, el tamaño del tumor y el número de células Treg en bazo de ratones que desarrollan ET sólido. Además, la administración de Ca10 exógeno en ratones aumenta significativamente el número de células Treg esplénicas. Por otro lado, también hemos demostrado que la diferenciación de monocitos humanos en presencia de Ca10 genera DCs tolerogénicas con una mayor capacidad de polarizar células Treg y una menor inducción de células T productoras de IFNγ en comparación con las DCs generadas en ausencia de Ca10.

En resumen, en esta Tesis Doctoral descubrimos nuevos mecanismos moleculares mediante los cuales la combinación de MV140 y V132 y el carbohidrato tumoral Ca10 modulan el fenotipo y la función de DCs humanas y su capacidad para polarizar respuestas de célula T.

Hemos mostrado que MV140/V132 promueve DCs que generan células Th1, Th17 y Treg. V132 induce inmunidad entrenada en monocitos humanos, lo que aumenta significativamente las respuestas pro-inflamatorias frente a una segunda estimulación con MV140. Además, nuestros resultados *in vivo* demuestran la relevancia potencial de estos hallazgos en diferentes modelos preclínicos de ratón.

Por otro lado, hemos dilucidado un nuevo mecanismo molecular por el cual el carbohidrato Ca10 induce DCs tolerogénicas y reprograma la diferenciación de monocitos hacia DCs tolerogénicas con capacidad de polarizar células Treg supresoras. También hemos demostrado, por primera vez, la inducción de células Treg en ratones portadores del ET sólido, lo que se correlaciona positivamente con los niveles de Ca10 circulantes. Apoyando estos datos, la inmunización de ratones con Ca10 aumenta el número de células Treg esplénicas.

Los resultados presentados en esta Tesis Doctoral sobre los mecanismos moleculares implicados en los efectos beneficiosos de nuevas preparaciones sublinguales microbianas (MV140 y V132) para GUTIs, podrían contribuir al desarrollo de nuevos tratamientos para infecciones recurrentes. Además, nuestros datos sobre el mecanismo por el cual carbohidratos tumorales inducen DCs tolerogénicas y promueven células Treg supresoras, podrían contribuir al desarrollo de nuevas vías de investigación con implicaciones clínicas importantes en el cáncer.

ABSTRACT

Dendritic cells (DCs) are professional antigen-presenting cells that play a key role in the orchestration of proper immune responses by linking innate and adaptive immunity. DCs exhibit the dual capacity to promote potent immune responses against potentially dangerous pathogens or to generate tolerance against self- and non-self-innocuous antigens, a process that must be tightly regulated and that strongly depends on the encountered antigens and surrounding environmental signals. Alterations in the regulation of this vital DC's function might well lead to different immune-mediated diseases such as recurrent infections, cancer, autoimmunity or allergy.

Infections and cancer represent major healthcare problems with increasing prevalence and high socio-economic impact worldwide. Genitourinary tract infections (GUTIs), including recurrent urinary tract infections (RUTIs) and recurrent vulvovaginal candidiasis (RVVCs), are among the most common bacterial and fungal infectious diseases significantly impairing patients' quality of life. Antibiotics and antifungals remain the mainstay of treatment for these infections, respectively. However, their overuse is associated with microbial resistances and microbiota deleterious effects. Trained immunity-based vaccines (TIbV) are vaccine formulations able to induce trained immunity, thus conferring protection not only against the antigens contained in the vaccine formulation but also against heterologous infections. TIbV have been regarded as potential novel suitable strategies for recurrent infections. Sublingual vaccine MV140 is a whole heat-inactivated polybacterial preparation composed of 75% Gram-negative bacteria (25% *Escherichia coli*, 25% *Proteus vulgaris* and 25% *Klebsiella pneumoniae*) and 25% Gram-positive bacteria (*Enterococcus faecalis*). MV140 shows clinical efficacy for RUTIs acting on DCs promoting Th1, Th17 and IL-10 immune responses. V132 is a sublingual whole heat-inactivated *Candida albicans* formulation for RVVCs. Given the tight relationship between RUTIs and RVVCs, novel therapeutic strategies combining MV140 and V132 might well represent a potential suitable alternative for GUTIs. However, prior to clinical translation, a better understanding of the molecular mechanisms underlying the mode of action of this potential combination is highly needed.

During tumor development, malignant transformation of cancer cells is frequently accompanied by aberrant changes in tumor-associated surface glycoconjugates, which have a strong influence on tumor progression. These tumoral carbohydrates inhibit anti-cancer immunity through different mechanisms including the expansion of regulatory T (Treg) cells. The aberrant tumoral carbohydrate A10 (Ca10) is a cell-surface carbohydrate derived from Ehrlich tumor (ET) cells defined by the binding of the

monoclonal antibody A10. Ca10 is spontaneously released from ET cells and can be detected in the serum of solid ET-bearing mice, which might represent a tumor escape mechanism. However, a deeper mechanistic knowledge at the cellular and molecular level on the potential implications of the carbohydrate Ca10 in tumor progression is highly demanded.

Considering all these aspects, the global aim of this Doctoral Thesis is to investigate the underlying molecular mechanisms by which the bacterial (MV140) and fungal (V132) sublingual vaccines used for recurrent GUTIs as well as the aberrant tumoral carbohydrate Ca10 from ET cells, regulate the phenotype and functional properties of human DCs, including their ability to polarize T cell responses.

In this Thesis, we demonstrated that the combination of MV140 and V132 in a single formulation acts on human DCs promoting the generation of IFNγ- and IL-17A-producting T cells as well as Treg cell responses. Mechanistically, MV140/V132 activates NF-κB, MAPKs and mTOR signalling pathways, induces a metabolic shift towards glycolysis and lactate production, and histone methylation within cytokine-related genes in human DCs. Splenic $CD4^+$ T cells from mice sublingually immunized with MV140/V132 display enhanced proliferation upon *in vitro* stimulation with the antigens contained in the formulation as well as with the non-related antigen, phytohaemagglutinin. Additionally, sublingual immunization of mice with MV140/V132 generates IgG and IgA antibodies against the specific components of the vaccine. We also reported that V132 induces training of human monocytes, thus enhancing pro-inflammatory cytokine production upon MV140 secondary stimulation. V132-trained monocytes display a metabolic reprogramming towards an increase of glycolysis and oxidative phosphorylation and epigenetic rewiring that enhances the transcription of the pro-inflammatory genes *IL6* and *TNFA*. *In vivo*, immunization of mice with V132 induces training in peritoneal cells and splenocytes, which contribute to potentiate IL-17 responses in MV140-treated mice upon *in vitro* stimulation with MV140.

In this Thesis, we also demonstrated that the tumoral carbohydrate Ca10 promotes tolerogenic human DCs with the capacity to polarize highly suppressive functional Treg cells. The mechanisms involved in such effect partially depend on PD-L1, IL-10, IDO, mTOR pathway and glycolysis. We also showed that the heparan sulfate-related proteoglycan contained in the carbohydrate structure of Ca10 is essential for the induction of tolerogenic DCs with capacity to polarize Treg cells. In solid ET-bearing mice, we observed positive correlations between Ca10 serum levels, tumor size and splenic Treg cells. In addition, the administration of Ca10 to mice significantly increases the number of splenic Treg cells. We also reported that the differentiation of human

monocytes into DCs in the presence of Ca10 generates tolerogenic DCs with the capacity to polarize higher Treg cells and lower IFNγ-producing T cells than conventional DCs generated in the absence of Ca10.

In summary, in this Doctoral Thesis we have uncovered previously unknown molecular mechanisms by which the combination of M140 and V132 and the tumoral carbohydrate Ca10 regulate the phenotype and function of human DCs and their capacity to polarize T cell responses.

We reported that MV140/V132 endorses DCs with the capacity to generate Th1, Th17 and Treg cells. Remarkably, we also demonstrated that V132 induces trained immunity in human monocytes, which significantly enhances their pro-inflammatory responses upon second challenge with MV140. We also showed the potential relevance of our findings in different *in vivo* preclinical mouse models.

We also provided novel molecular insights into the mechanisms by which the heparan sulfate-related structure of carbohydrate Ca10 imprints tolerogenic DCs and reprograms monocytes into tolerogenic DCs with the capacity of polarizing highly suppressive Treg cells. We demonstrated, for the first time, the *in vivo* induction of Treg cells in mice bearing solid ET, which was positively correlated with the serum levels of circulating Ca10. Supporting these data, *in vivo* immunization of mice with Ca10 increases the number of splenic Treg cells.

The data presented in this Doctoral Thesis shed light into novel molecular mechanisms involved in the beneficial effects of novel microbial sublingual preparations (MV140 and V132) for GUTIs, which might well contribute to pave the way for the development of new interventions for recurrent infections. In addition, our findings regarding the fundamental knowledge on how tumoral carbohydrate structures imprint tolerogenic DCs and promote suppressive Treg cells might well contribute to open new avenues of research with important clinical implications in cancer.

INTRODUCTION

1. The immune system

1.1. An overview of the immune response and immunological memory

The immune system is a complex network of cells, tissues, organs and molecules that displays the ability to develop proper immune responses against a wide range of potentially dangerous pathogens while maintaining, at the same time, a state of tolerance towards self- and non-self-harmless antigens. The immune system has several tightly regulated mechanisms to perform this essential function called the 'immune response'. Dysregulation of such mechanisms might well lead to a plethora of immune-mediated diseases such as susceptibility to infections, cancer, allergy, autoimmune diseases or organ rejection (1, 2).

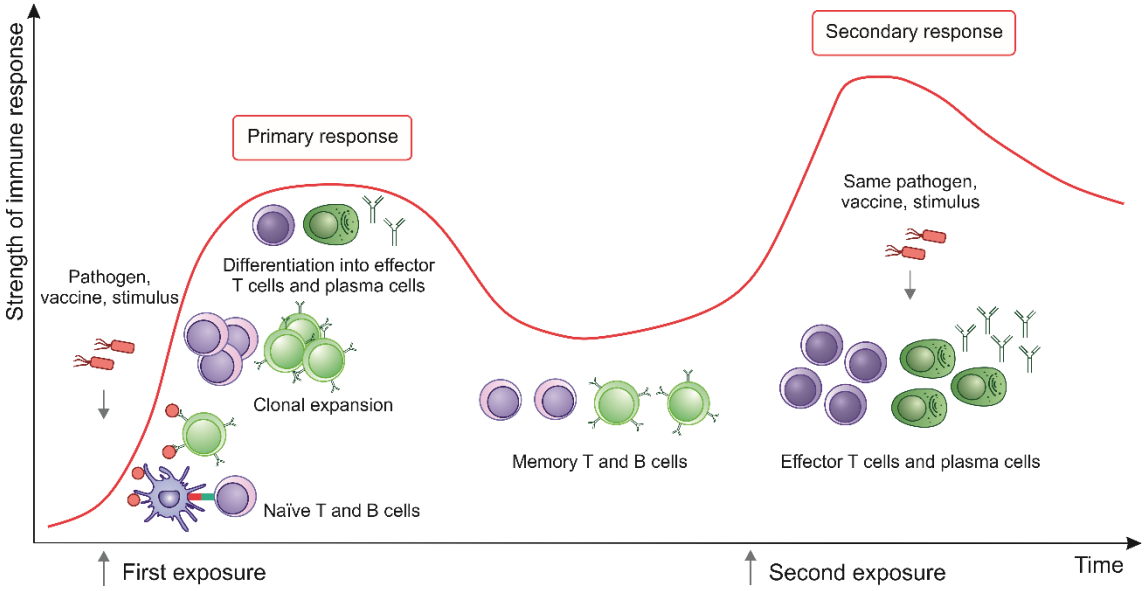

Figure 1. Primary and secondary adaptive immune response. In a primary response to a pathogen, vaccine or stimulus, naïve T and B cells are activated. The recognition of the antigen is followed by a clonal expansion in an antigen-specific manner and differentiation into effector T cells and plasma cells, thus generating memory T and B cells. In a second encounter with the same pathogen, vaccine or stimulus, memory T and B cells are able to respond more potently and efficiently, thus contributing to pathogen clearance. Adapted from *Abbas, A.K. (2018). Cellular and Molecular Immunology (9th edition). Elsevier.*

Classically, host immunity is divided into the innate and adaptive immune responses (1, 3). Innate immunity is mediated by innate immune cells such as phagocytic cells (neutrophils and macrophages), dendritic cells (DCs) and natural killer (NK) cells; physical and chemical barriers (epithelia and antimicrobial substances); as well as humoral components such as complement system and cytokines (1). Adaptive immune response is relied on T cells and B cells, able to produce immunoglobulins (Ig) or antibodies (2). During an infection, innate immune cells provide the first and early line of defense acting very rapidly after a pathogen or toxin is encountered (1, 3). Lately,

activation of adaptive immune response takes place allowing the specific recognition and elimination of the pathogen. After the recognition of the antigen, adaptive immune cells proliferate in an antigen-specific manner, thus generating a pool of long-lasting memory cells. Upon a second encounter with the same pathogen, adaptive memory cells can respond much more potently and efficiently, thus contributing to pathogen clearance and to avoid infection (**Figure 1**) (2).

Although the immune memory has been exclusively associated with the adaptive immune system, in the last decades several studies convincingly demonstrated that innate immune cells also exhibit memory. First evidence of innate memory was described in mice after sublethal *Candida albicans* infection which confers protection against reinfection by a T and B cell independent mechanism (4). Then, an enhanced cytokine release after *in vitro* stimulation with non-related pathogens was reported in peripheral blood mononuclear cells (PBMC) from individuals previously vaccinated with the *Bacille Calmette-Guérin* (BCG) (5). This enhanced responsiveness was defined as trained immunity by Netea and colleagues in 2011. Trained immunity can be defined as the process by which innate immune cells display long-term functional reprogramming after an encounter with a primary stimulus, resulting in an enhanced immune response against a secondary related or unrelated challenge (6-8). After recognition of the first stimulus, innate immune cells (monocytes, macrophages, NK cells, neutrophils, DCs and innate lymphoid cells, ILCs), bone marrow progenitors and even non-immune cells (such as epithelial and endothelial cells) undergo metabolic and epigenetic rewiring, the two central molecular programs of trained immunity induction (4, 9-16). The long-term reprogramming of bone marrow progenitors allows the establishment of innate memory response that can be maintained for weeks or months (15, 16). Importantly, after encounter with the first pathogen or antigen, trained cells return to the steady state without producing cytokines or chemokines. Remarkably, and in contrast to adaptive immunity, innate immune memory is triggered by a second encounter with the same stimulus or a different one. Therefore, trained immunity confers a broader protection against heterologous pathogens (**Figure 2**).

Several ligands of microbial origin have been described as trained immunity inducers such as β-glucan (located on the cell wall of fungi), BCG (live attenuated pathogen) or MV130 (whole heat-inactivated pathogens); but also endogenous ligands including oxidized low-density lipoprotein, uric acid or catecholamines, among others, are able to induce a trained phenotype (4, 9, 17-20).

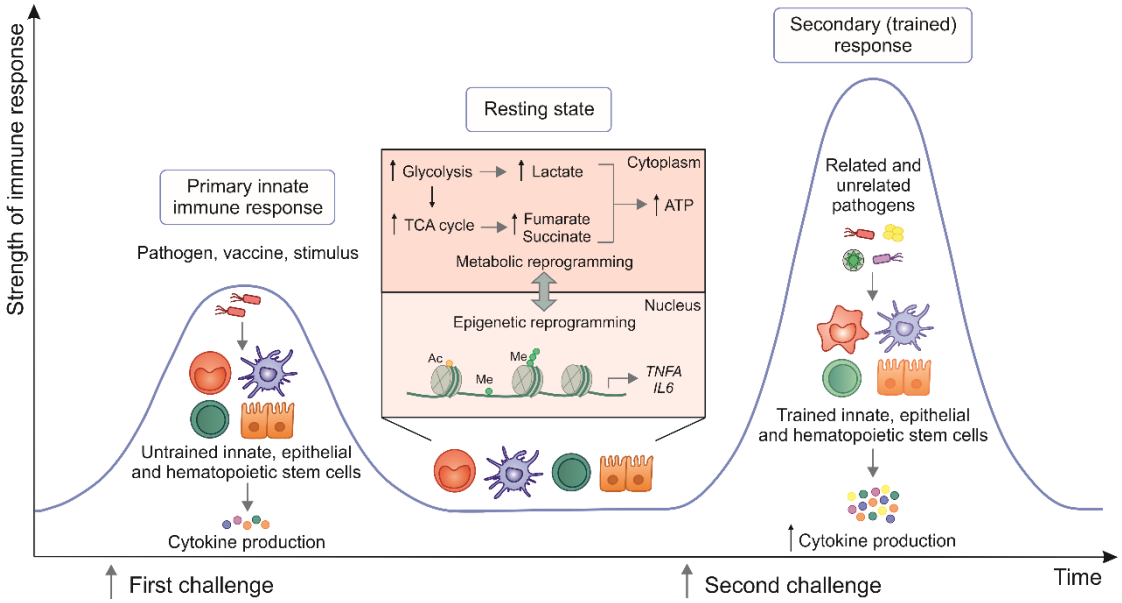

Figure 2. Induction of trained immunity. First exposure to certain pathogens, vaccines and stimuli promotes long-term functional, metabolic and epigenetic reprogramming of innate, epithelial and hematopoietic stem cells. Trained immunity induces enhanced responses after a second exposure with related or unrelated pathogens, thus conferring heterologous protection. Ac, acetylation; ATP, adenosine triphosphate; Me, methylation; TCA, tricarboxylic acid. Adapted from *From trained immunity in allergy to trained immunity-based allergen vaccines. Clin Exp Allergy, 2022;00:1-11.*

1.2. Dendritic cells as the link of innate and adaptive immune responses

DCs are professional antigen-presenting cells (APCs) crucial in the orchestration of proper immune responses by linking innate and adaptive immunity (21, 22). DCs were discovered by Steinman and Cohn in 1973 in peripheral lymphoid organs of mice (23). DCs are located in all peripheral tissues, primary and secondary lymphoid organs and blood (24). Depending on the antigens and the received signals in the peripheral tissues and lymph nodes, DCs display the dual capacity to induce appropriate immune responses against pathogens or to generate tolerance against self- and non-self-innocuous antigens (21, 25, 26). To perform this essential functions, DCs recognize and internalize encountered antigens within the tissues, which are processed and then presented to naïve T cells in the lymph nodes to initiate proper adaptive immune responses depending on the inflammatory context and nearby environmental signals (27-29).

In peripheral tissues under homeostatic non-inflammatory conditions, DCs display an immature phenotype with reduced expression of mature and co-stimulatory markers CD80, CD86, CD83 and CD40, reduced cytokine secretion, high phagocytic and processing antigen capacity and limited capacity to stimulate T cells (30-32). On sensing surrounding signals such as pathogen infection, vaccination, injury or tumor

microenvironment (TME) components, DCs mature and express co-stimulatory molecules mentioned above, and chemokine receptors. The main one is C-C chemokine receptor 7 (CCR7) which drives the migration of mature DCs into lymph nodes to present the processed antigens to naïve T cells (33, 34). The processed antigen is presented in a major histocompatibility complex (MHC) class I and class II molecules on the surface of DCs. MHC class I molecules present peptides derived from endogenous antigens to $CD8^+$ cytotoxic T lymphocytes (CTLs), whereas MHC class II molecules bind peptides derived from exogenous antigens that are recognized by $CD4^+$ T cells (T helper, Th). However, DCs also display the capacity to present extracellular antigens to $CD8^+$ T cells by MHC class I molecules under certain circumstances in a process termed 'cross-presentation' (28, 35, 36). Traditionally, it has been widely accepted that immature or partially mature DCs induce regulatory T (Treg) cells in order to promote tolerance, whereas mature DCs polarize different types of pro-inflammatory effectors T cells. However, recent studies have demonstrated that Treg cells are also generated by fully mature DCs under specific conditions (37-40).

DCs recognize a wide variety of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) through different pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) and C-type lectin receptors (CLRs) (41, 42). **TLRs** are transmembrane receptors that mediate the recognition of microbial components. In humans, TLR1, 2, 4, 5, 6 and 10 expressed on cell surface recognize diverse surface microbial components (lipopolysaccharide, LPS; or flagellin); and TLR3, 7, 8 and 9 located in endoplasmic reticulum and endosomal membranes detect nucleic acids (43, 44). **NLRs** are cytoplasmic receptors that recognize ligands from pathogens (peptidoglycan, flagellin, viral RNA or fungal hyphae), cell components (cholesterol or ATP), or other environmental stimuli (UV radiation or alum) (45, 46). Other type of cytoplasmic receptors are **RLRs** which sense viral and host-derived RNAs (47, 48).

CLRs are transmembrane receptors containing at least one C-type lectin domain which recognize, in a calcium-dependent manner, a wide variety of carbohydrate structures contained in pathogens and host cells (49, 50). CLRs mediate multiple functions such as phagocytosis, cell adhesion and induction of pro-inflammatory or tolerogenic responses (50). Triggering of CLRs drives the induction of adaptive immune responses by enhancing antigen presentation as well as priming different subsets of T effector cells (51). Depending on the intracellular signalling motifs and the downstream signalling pathways, CLRs can be divided into three groups: (i) Spleen tyrosine kinase (Syk)-coupled CLRs including CLRs containing immunoreceptor tyrosine-based

activating motif (ITAM) (such as dectin-2 or Mincle) and CLRs containing hemi-ITAM (such as dectin-1), (ii) immunoreceptor tyrosine-based inhibitory motif (ITIM)-coupled CLRs (such as DC immunoreceptor, DCIR), and (iii) CLRs without ITIM or ITAM (such as mannose receptor or DC-specific intercellular adhesion molecule 3-grabbing non-integrin, DC-SIGN) (**Figure 3**) (52). ITAM-coupled CLRs interact with ITAM-containing adaptor proteins, whereas hemITAM-coupled CLRs contain a ITAM motif in their cytoplasmic tail (52). Both CLRs lead to Syk activation which drives the activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) signalling pathways, among others (50, 52, 53).

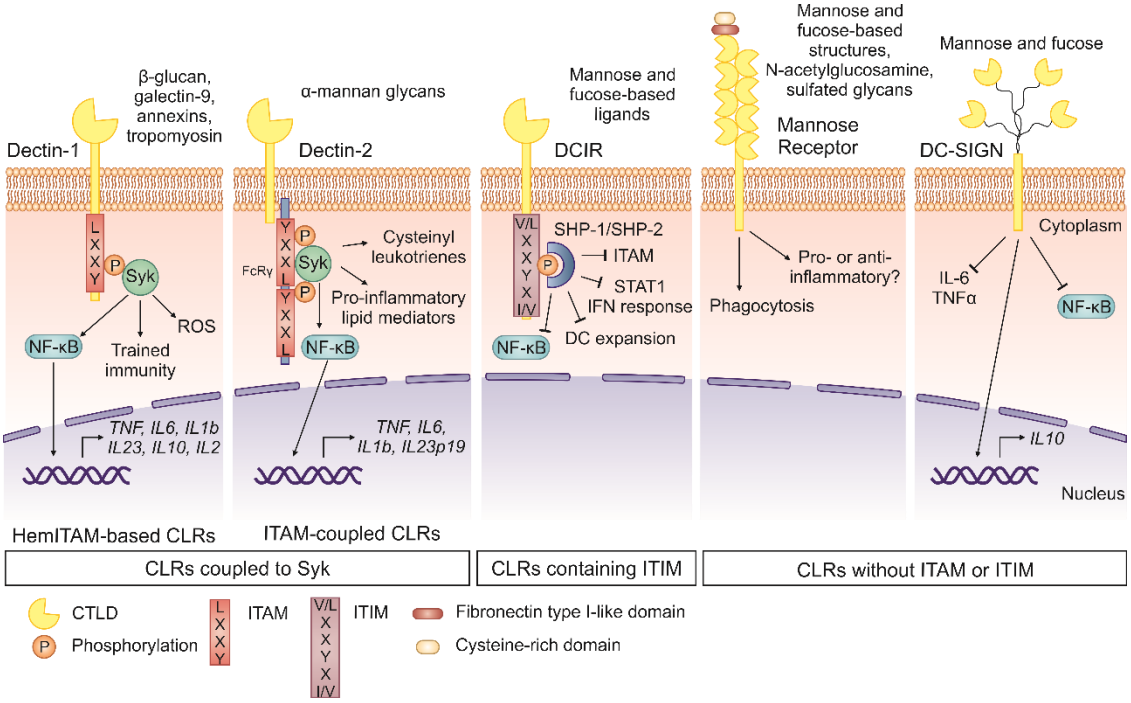

Figure 3. Main CLRs-induced signalling pathways. After ligand recognition, dectin-1 induces phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) which recruits spleen tyrosine kinase (Syk). Subsequently, activation of nuclear factor κB (NF-κB) signalling pathways results in the induction of cytokine gene expression. Dectin-1 is also involved in reactive oxygen species (ROS) production and the induction of trained immunity. Activation of dectin-2 induces signalling pathways through ITAM-containing the adaptor molecule (e.g. Fc receptor γ-chain, FcRγ). The phosphorylation of ITAM recruits Syk inducing NF-κB pathway which modulates cytokine gene expression. Dectin-2-mediated activation of Syk is involved in the production of pro-inflammatory lipid mediators and cysteinyl leukotrienes. Activation of DC immunoreceptor (DCIR) leads the phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIM). The recruitment of the phosphatases SH2-domain-containing protein tyrosine phosphatase 1 (SHP1) or SHP2 inhibits NF-κB and STAT1-type I IFN signalling pathways, limits DC expansion and downregulation of ITAM. Mannose receptor and DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) lack ITAM or ITIM domains. Mannose receptor mediates phagocytosis, but its implication in pro- or anti-inflammatory responses is still not clear. DC-SIGN induces the inhibition of the NF-κB pathway which mediates the downregulation of IL-6 and TNFα production, whereas favouring *IL10* gene expression. Adapted from *C-type Lectin Receptor Mediated Modulation of T2 Immune Responses to Allergens. Curr Allergy Asthma Rep, 2022. In press*.

Human DCs have initially been classified according to the expression of surface markers. However, other properties such as the transcriptome program, the morphology, the migratory capacity and the ability to polarize naïve T cells, can be used to identify DC subsets (54-57). Traditionally, human DCs are broadly classified into conventional or myeloid DCs (cDCs or mDCs, respectively) and plasmacytoid DCs (pDCs). mDCs can be divided in $CD141^+$ mDCs (mDC1) or $CD1c^+$ mDCs (mDC2) (24, 58). Recently, deeper studies in the transcriptome program and ontogeny have allowed the identification of two subtypes of mDC2: DC2 and DC3. In addition, a novel DC subset has been defined: AXL^+ $Siglec6^+$ DCs (AS DCs) (**Table 1**) (57, 59, 60).

In humans, DCs are derived from $CD34^+$ granulocyte–macrophage DC progenitors (GMDPs) generated in the bone marrow (58). GMDPs expressing high levels of IRF8 give rise plasmacytoid DC precursor (pre-pDC) or conventional DC precursor (pre-cDC). Pre-cDC generates cDC1 and cDC2 subsets. In contrast, $IRF8^{low}$ GMDPs give rise to DC3 and monocytes (59, 61). Differentiation of all DC subtypes is mainly dependent of FMS-like tyrosine kinase ligand 3 (FLT3L or CD135) (58). DCs are characterized by the expression of MHC class II molecules (HLA-DR) and the absence of linage markers as CD3 (T cells), CD19/20 (B cells) and CD56 (NK cells) (62). Mouse counterparts of human DCs have been extensively studied (**Table 1**) (56, 63).

1.2.1. Human myeloid dendritic cells (mDCs)

mDCs or cDCs are defined as $CD11c^+$ and MHC class II ($HLA\text{-}DR^+$). These DCs are located in lymphoid tissues and blood, and are the primary subset involved in priming of potent T cells responses. mDCs can be divided into two main subsets, mDC1 or mDC2 (21).

mDC1 or CD141/BDCA-3^+ mDCs can also be identified by the expression of Clec9A. mDC1 have the ability to induce cellular immunity against intracellular pathogens and tumors due to their efficient processing and cross-presentation to activate $CD8^+$ T cells. In addition, mDC1 are also involved in driving $CD4^+$ Th1 cell responses. Mouse cDCs are the equivalent to human mDC1, which can be divided into $CD8\alpha^+$ resident DCs and $CD103^+$ migratory DCs (**Table 1**) (21, 55, 64).

mDC2 or CD1c/BDCA-1^+ mDCs comprise a heterogeneous population characterized by the CD1c expression. The use of single-cell RNA-sequencing has allowed the identification of two different subtypes of mDC2: DC2 and DC3. DC2 are differentiated as $CD32B^+$, $CD5^{high}$, $CD11b^+$ and MHC class II ($HLA\text{-}DR^{high/+}$) by flow cytometry. DC2 are highly efficient in MHC class II-mediated antigen presentation for priming of $CD4^+$ T cell responses and drive the generation of Th2, Th17 and Treg cells.

DC3 have common transcriptomic profile with DC2 and monocytes. DC3 express CD163, CD36, CD14, S100A8 and S100A9, and low expression of HLA-DR (**Table 1**) (55, 57, 59, 65, 66).

Table 1. Phenotype and function of human DC subsets and equivalent mouse DC subsets.

	mDC1	mDC2		pDC	AS DC	moDC
		DC2	DC3			
Phenotype	$CD11c^+$ $CD141^+$ $HLA\text{-}DR^+$ $Clec9A^+$ $XCR1^+$	$CD11c^+$ $CD1c^+$ $HLA\text{-}DR^+$ $CD11b^+$ $CD5^+$ $CD32B^+$	$CD11c^+$ $CD1c^+$ $HLA\text{-}DR^+$ $CD14^{low/+}$ $CD5^-$ $CD163^+$ $CD36^+$ $S100A8/9^+$	$CD11c^-$ $HLA\text{-}DR^+$ $CD123^+$ $CD303^+$ $CD304^+$	$CD123^+$ $CD303^+$ AXL^+ $Siglec6^+$ $CD2^+$ $CD5^+$	$CD11c^+$ $HLA\text{-}DR^+$ $CD11b^+$ $CD1c^+$ $CD14^+$ $S100A8/A9^+$
PRRs	TLR1, 3, 6, 8, 10, STING, CLEC12A	TLR1, 2, 4, 5, 6, 8, RLR, NLR, CLRs	Not well defined	TLR7, 9, RLR, STING, CLEC12A	Not well defined	TLR1, 2, 3, 4, 7, 8
Functions	Cross-presentation, $CD4^+$ and $CD8^+$ T cell priming, induction of Th1 cells	$CD4^+$ T cell priming, induction of Th1, Th2, Th17 and Treg cells, cross-presentation	$CD4^+$ T cell priming, induction of Th1 and Th17 cells	Anti-viral responses, cross-presentation, $CD4^+$ T cell priming, induction of Th1 and Treg cells	$CD4^+$ T cell stimulation	Inflammation, $CD4^+$ and $CD8^+$ T cell priming, induction of Th1, Th2, Th17, Treg cells
Cytokines	IL-12, TNF, IL-6, I IFN, III IFN	IL-1β, IL-6, IL-12, IL-23, IL-10, TNF, TGF-β	IL-12, IL-23, IL-1β	I IFN, III IFN, TNF	Not I IFN production	IL-1β, IL-6, IL-10, IL-12, IL-23, TNF, iNOS
Equivalent mouse subsets	cDC1: $CD11c^+$ $MHC\text{-}II^+$ $Clec9A^+$ $XCR1^+$ $CD205^+$ $CD8\alpha^+$ (resident) or $CD103^+$ (migratory)	cDC2: $CD11c^+$ $MHC\text{-}II^+$ $CD11b^+$	Not well defined	pDCs: $CD11c^{+/low}$ $MHC\text{-}II^+$ $B220^+$ $SiglecH^+$ $CD317^+$ $CCR9^+$	Transitional DC: $CX3CR^{high}$	moDCs: $CD11c^+$ $MHC\text{-}II^+$ $CD11b^+$ $CD14^+$ $Ly6C^+$ $CD64^+$

Adapted from references (21, 57, 59, 60, 67).

1.2.2. Human plasmacytoid dendritic cells (pDCs)

pDCs are defined as $CD123^+$, CD303/BDCA-2^+, CD304/ BDCA-4^+ and $HLA\text{-}DR^+$. pDCs are mainly involved in anti-viral responses upon recognition of nucleic acids through TLR7 and TLR9, while are potent producers of type I interferons (IFN) (68, 69). Furthermore, pDCs are able to present antigens to $CD4^+$ T cells inducing forkhead box P3 $(FOXP3)^+$ Treg cells with suppressive capacity (**Table 1**) (39, 70, 71).

AS DCs are a subset of pDCs identified by single-cell RNA-sequencing as $CD123^+$, $CD303^+$, $CD11c^{-/low}$, AXL^+, $Siglec6^+$, $CD2^+$ and $CD5^+$. AS DCs display the ability to stimulate allogeneic $CD4^+$ and $CD8^+$ T cell proliferation, without type I IFN production upon TLR9 stimulation. AS DCs can be *in vitro* differentiated into mDC2 and therefore,

they are considered a 'transitional' population between pDCs and mDC2 (57, 59). The mice counterpart of human AS DCs was also identified with common features to pDCs and cDCs (**Table 1**) (67).

1.2.3. Human monocyte-derived dendritic cells (hmoDCs)

Monocytes represents around 10% of the total human PBMC. The major function of monocyte is host defense against pathogens. Under inflammatory conditions, monocytes are rapidly recruited from the blood into tissues where they are differentiated into DCs (72-75). *In vitro,* monocytes are differentiated into human monocyte-derived DCs (hmoDCs) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (76, 77). HmoDCs display an immature phenotype with a low antigen-presenting capacity. However, under the presence of microbial signals, TME components, allergens or other stimuli, hmoDCs maturate and acquire an immunogenic or tolerogenic phenotype, while increasing their antigen-presenting capacity and inducing $CD4^+$ and $CD8^+$ T cell activation and differentiation (38, 74, 75, 78-82).

1.3. Mechanisms involved in polarization of naïve $CD4^+$ T cells

As previously described, DCs uptake and process antigens to present them to naïve T cells (28). The activation of T cells requires three distinct signals: (i) antigen presentation, (ii) costimulation by surface molecules, and (iii) soluble factors (polarizing cytokines) (**Figure 4**) (21, 28). The recognition of the antigen ensuring the specificity requires that T-cell receptor (TCR) of naïve $CD4^+$ T cells interacts with the peptide-MHC class II complex displayed by DCs in a restricted manner (signal 1). The requirement for additional stimuli triggered by APCs (signals 2 and 3) ensures proper activation and determines the polarization of adaptive immune cells. DCs express costimulatory molecules that are recognize by $CD4^+$ T cells (signal 2). Among these costimulatory molecules, CD80 and CD86 expressed on DCs interact with CD28 to induce T cell activation (83). Other relevant costimulatory pathways that control the activation of T cells is the binding of OX40L or CD40 on DC surface and T-cell expressed OX40 or CD40L, respectively (21). In contrast, programmed cell death-1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA4) limit T cell effector activity when interacting with programmed death-ligand 1 (PD-L1) and PD-L2, or CD80 and CD86 on DCs, respectively (84, 85). Indoleamine 2,3-dioxygenase (IDO) expressed by DCs promotes tolerance by metabolizing L-tryptophan, which is a key amino acid involved in T cell activation (86). Finally, cytokines or soluble molecules released by DCs complete T cell activation and polarization (signal 3) (**Figure 4**) (21).

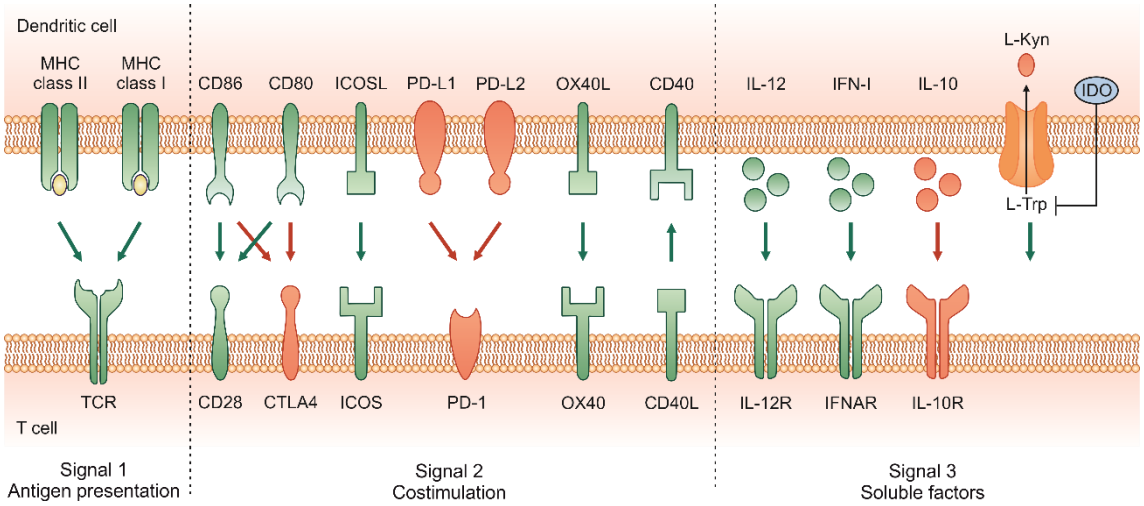

Figure 4. Signals for T cell activation. The activation of T cells requires antigen recognition (signal 1), costimulation (signal 2) and soluble factors, mainly cytokines (signal 3). Positive signalling (green arrows and receptors) and inhibitory mechanisms (red arrows and receptors) are represented. CTLA4, cytotoxic T lymphocyte antigen 4; ICOS, inducible T-cell costimulator; ICOSL, inducible costimulator ligand; IDO, indoleamine 2,3-dioxygenase; IFNAR, type I interferon receptor; IL-10R, IL-10 receptor; IL-12R, IL-12 receptor; Kyn, kynurenine; MHC, major histocompatibility complex; PD-1, programmed cell death-1; PD-L1 or PD-L2, programmed death-ligand 1 or 2; TCR, T cell receptor; Trp, tryptophan. Adapted from *Dendritic cells in cancer immunology and immunotherapy. Nat Rev Immunol, 2020; 7-24*.

The ability of DCs to induce different effector T or Treg cell responses is conditioned by the polarizing cytokines and the signals (28). $CD4^+$ T cells acquire specific phenotypes but have certain plasticity to adapt their functions in response to changing circumstances (87). Classification of effector $CD4^+$ T cells relies on surface markers, transcription factors and cytokine production (28, 88). Th1, Th2 and Th17 cells are the main subsets involved in host immune responses against pathogens, while Treg cells are one of the key mechanisms to induce tolerance and control excessive activation of the immune system (**Figure 5**) (88, 89).

1.3.1. T helper 1 (Th1) cells

Th1 cells are essential for orchestrating host defense against intracellular virus, bacteria and protozoa. IL-12 and TNFα produced by DCs drive Th1 differentiation and activate T-box transcription factor (T-bet) in $CD4^+$ T cells. T-bet is the master transcription factor for Th1-cell differentiation, which directly regulates IFN-γ production. IFN-γ is released by activated Th1 cells and induces T-bet expression, thus acting as a positive feedback for Th1 differentiation. IL-12 and IFN-γ also activate transcription factors STAT4 and STAT1, respectively (88, 90). IFN-γ-producing Th1 cells activate macrophages and promote cytotoxic activities of NK cells and $CD8^+$ T cells to kill infected cells, as well as the Ig-class-switching (**Figure 5**) (91).

1.3.2. T helper 2 (Th2) cells

Th2 cells are critical for mediating immune responses against helminths, venoms and in the pathogenesis of asthma and allergic diseases. IL-4 activates STAT6 while inducing polarization of Th2 cells. The master regulator of Th2 differentiation, GATA binding protein 3 (GATA3), leads the production of IL-4, IL-5 and IL-13 by Th2 cells, which contribute to IgE production, tissue migration of eosinophils or the disruption of the epithelial barrier integrity (91). Interestingly, GATA3 strongly suppress Th1- and Th17-associated gene expression (**Figure 5**) (88, 92).

1.3.3. T helper 17 (Th17) cells

Th17 cells play a key role against extracellular bacteria and fungi, and are also involved in autoimmunity. Together with IL-6, IL-1β and IL-23 produced by DCs, transforming growth factor β (TGF-β) promotes Th17 cell differentiation. Retinoic acid-related orphan receptor γt (RORγt) is the master transcription factor for Th17 cell differentiation (88, 90, 92). Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22. By inducing cells to produce cytokines and chemokines, IL-17A and IL-17F mediate the recruitment and activation of neutrophils. IL-21 favors B cell proliferation, differentiation and survival, as well as cytotoxic activities and proliferation of $CD8^+$ T cells, NK cells and NKT cells. IL-22 is involved in immune responses at the mucosal barriers by stimulating the production of antimicrobial peptides, chemokines and pro-inflammatory cytokines (**Figure 5**) (88, 91).

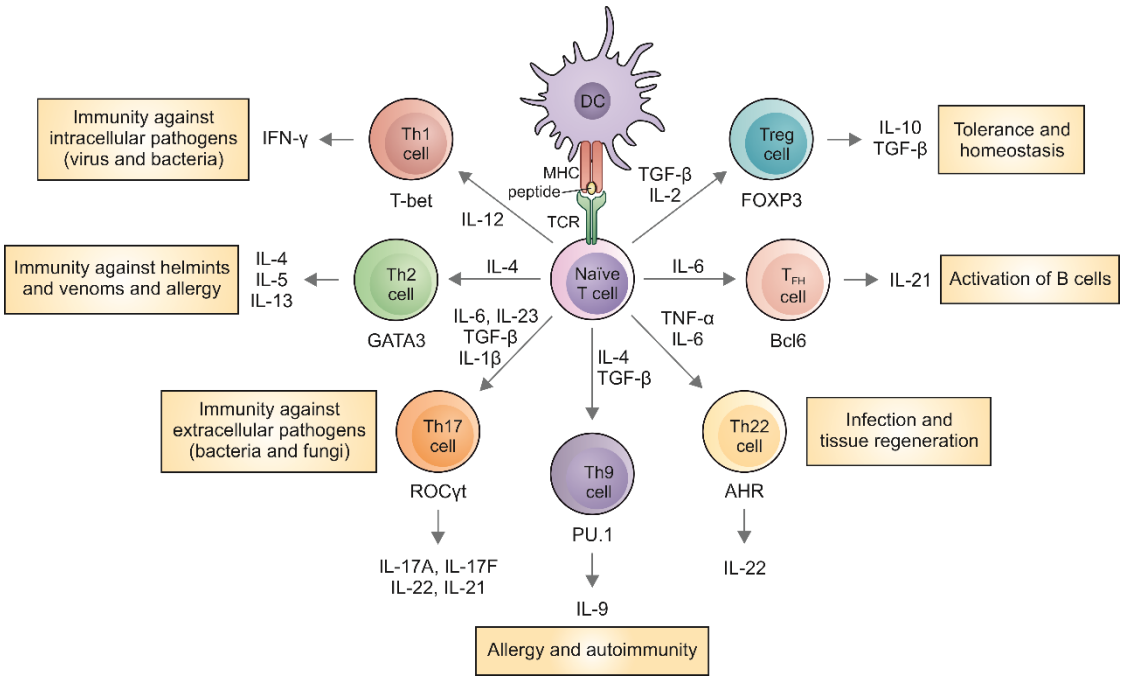

Figure 5. Differentiation of naïve T cell to functional T cell subsets. Adapted from *The development and in vivo function of T helper 9 cells. Nat Rev Immunol, 2015; 5:295-307.*

1.3.4. T helper 9 (Th9), 22 (Th22) and follicular helper (T_{FH}) cells

IL-4 and TGF-β drive Th9 cells differentiation that express the transcription factor PU.1 and produce IL-9. Th9 cells can exhibit anti-helminth activity and contribute to asthmatic disease or inflammatory bowel disease (93). Th22 cells are defined by IL-22 production and by the expression of the aryl hydrocarbon receptor (AHR). Th22 cells participate in the immune response against pathogens and tissue regeneration (94). T_{FH} cells promote humoral immunity within the germinal centers by favoring the differentiation of B cells into plasma cells. The transcriptional factor B-cell lymphoma 6 (Bcl6) drives T_{FH} cells, which can be defined by C-X-C chemokine receptor type 5 (CXCR5) and PD-1 expression, as well as IL-21 and IL-4 production (**Figure 5**) (95). In peripheral blood, circulating T_{FH} cells have been identified which also express CXCR5 and PD-1, but lack of Bcl6 expression. In addition, T follicular regulatory (T_{FR}) cells regulate the interaction between T_{FH} and B cells to prevent excessive activation or the development of allergy. T_{FR} cells express CXC5 and PD-1, but also FOXP3 (96, 97).

1.3.5. Regulatory T (Treg) cells

The generation and maintenance of functional Treg cells is a key mechanism to induce tolerance and prevent harmful excessive immune responses in many biological contexts such as autoimmunity, allergy, metabolic syndrome, transplantation, infection or cancer (98-101). There are two main subsets of Treg cells: (i) thymus-derived $CD4^+CD25^+FOXP3^+$ Treg (tTreg) cells, and (ii) peripherally-induced Treg (pTreg) cells generated outside the thymus upon antigenic stimulation, which can be divided into $FOXP3^+$ Treg cells, $FOXP3^-$ IL-10-producing Tr1 cells, and TGF-β-expressing Th3 cells (**Figure 6**) (37, 101, 102).

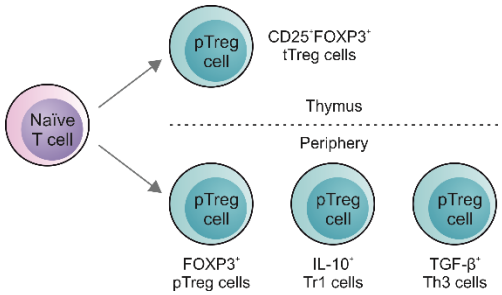

Figure 6. Different Treg cells subsets. Treg cells can be classified in thymus-derived Treg (tTreg) cells and peripherally-induced Treg (pTreg) cells. Adapted from *Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-β. Genes Immun, 2014; 15(8):511-20.*

In order to generate Treg cells, DCs produce a large number of soluble molecules (IL-10, TGF-β or IL-35), specific enzymes (IDO, or retinaldehyde dehydrogenase, RALDH) and metabolites (retinoic acid); and express different co-stimulatory molecules such as PD-L1, inducible costimulatory molecule ligand (ICOSL), CD86 or CD80 (37).

Treg cells are characterized by high expression of the α-subunit of the IL-2 receptor (CD25), lack expression of expression of the α-subunit IL-7 receptor (CD127) and expression of FOXP3 transcription factor, which is essential for Treg cell development and function (103). Treg cells induce suppression by using four main mechanisms, including secretion of suppressive cytokines (IL-10, TGF-β and IL-35), metabolic disruption mechanism (CD25; cyclic adenosine monophosphate, cAMP; histamine receptor 2, HR2; CD39 and CD73), cytolysis (granzyme A/B), expression of the co-inhibitory receptors (CTLA4; PD-1, T cell immunoglobulin and mucin domain-containing 3, TIM3; T cell immunoreceptor with immunoglobulin and ITIM domains, TIGIT; lymphocyte activation gene 3, LAG3; and neuropilin 1, NRP1) (**Figure 7**) (37, 104, 105).

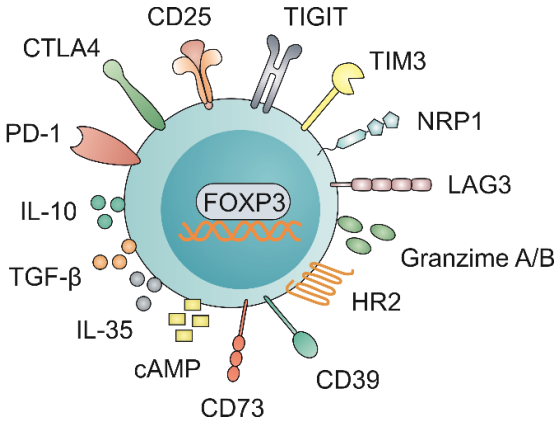

Figure 7. Suppressive mechanisms of Treg cells. Functional Treg cells suppress immune responses by using multiple mechanism including co-inhibitory receptors (cytotoxic T lymphocyte antigen 4, CTLA4; lymphocyte activation gene 3, LAG3; neuropilin 1, NRP1; programmed cell death-1, PD-1; T cell immunoglobulin and mucin domain-containing 3, TIM3; and T cell immunoreceptor with immunoglobulin and ITIM domains, TIGIT), secretion of suppression cytokines (IL-10, TGF-β and IL-35), cytolysis (granzyme A/B) and metabolic disruption mechanism (CD25; cyclic adenosine monophosphate, cAMP; histamine receptor 2, HR2; CD39 and CD73). Adapted from *Modulation of regulatory T cell function and stability by co-inhibitory receptors. Nat Rev Immunol, 2020; (11):680-693.*

2. Immunometabolism

2.1. An overview of the main metabolic pathways in the immune system

Immune cells regulate the activity of metabolic pathways to modulate the immune responses in a process termed as 'immunometabolism'. Changes in the metabolites of these pathways are produced to obtain energy to fuel and maintain intracellular processes, cellular growth and proliferation. The glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway, fatty acid oxidation, fatty acid synthesis and amino acid pathways are metabolic pathways regulated to link immune cell activity to their specific needs (**Figure 8**) (106).

- **Glycolysis**. Immune cells uptake extracellular glucose from the environment which is processed in the cytosol to generate pyruvate along with other products and only two molecules of ATP per unit of glucose. Although, the energetic balance is relatively inefficient, glycolysis allows the reduction of NAD^+ to NADH, which is used for the biosynthesis of multiple components in different anabolic pathways. Pyruvate can be reduced to lactate (lactic acid fermentation) or converted to acetyl coenzyme A (acetyl-CoA) to enter the mitochondria and fuel the TCA cycle. Glycolysis is upregulated when a faster production of ATP is required, which is essential for a rapid activation of immune cells (106).
- **TCA cycle and oxidative phosphorylation (OXPHOS)**. The TCA cycle (also known as the citric acid or Krebs cycle) takes place inside the mitochondria and receives acetyl-CoA from glucose-derived pyruvate or fatty acids, and other intermediates from different metabolic pathways. As a result, NADH and $FADH_2$ are generated that subsequently feed electrons into the electron transport chain to support OXPHOS, resulting a gradient for a highly efficient ATP production in the presence of oxygen. Per unit of glucose, thirty-two molecules of ATP are generated. This process provides basal requirements for energy and longevity in quiescent or non-proliferative cell conditions (106).
- **Pentose phosphate pathway (PPP)**. The PPP allows cells to obtain intermediates from glycolysis for the production of nucleotides and amino acid precursors. The activity of PPP is coupled with the generation of reducing equivalents of NADPH in the cytosol in order to maintain the cellular redox environment. PPP plays a key role in cell proliferation and survival (106).
- **Fatty acid synthesis**. Fatty acid synthesis takes place in the cytosol allowing the diversion of precursors from other metabolic pathways (glycolysis, TCA cycle and PPP) towards the production of the necessary lipids for cellular growth and proliferation (106).
- **Fatty acid oxidation**. Fatty acid oxidation allows cells to generate numerous products such acetyl-CoA, NADH and $FADH_2$ which can be employed to obtain energy. Oxidation starts in the cytosol and then, fatty acids diffuse into mitochondria allowing the production of numerous amount of ATP (106).
- **Amino acid synthesis**. Amino acids are linked with anabolic cellular signalling pathways, but they are also essential as precursors of different metabolites. For example, glutamine is required for supplying TCA cycle in actively proliferating cells; or tryptophan and arginine may be used to support proliferation and anabolic growth (106).

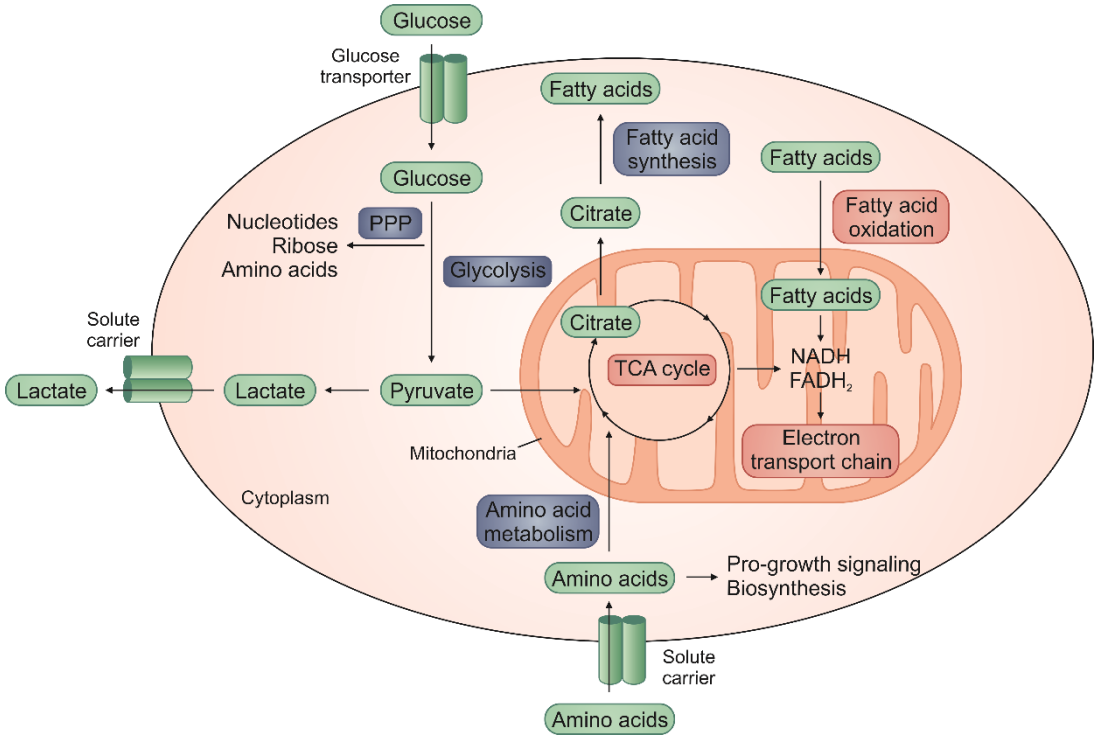

Figure 8. Six major metabolic pathways. Glycolysis, tricarboxylic acid (TCA) cycle, electron transport chain (oxidative phosphorylation), phosphate pathway (PPP), fatty acid oxidation, fatty acid synthesis and amino acid metabolism. Oxygen-dependent pathways are represented in red and pathways that not require oxygen are represented in blue. Adapted from *A guide to immunometabolism for immunologists. Nat Rev Immunol, 2016; 16(9):553-65.*

2.2. Metabolic control of dendritic cell function

Specific modifications of metabolic pathways regulate DC's function and activity, most notably by controlling the growth and survival needs of the cell as well as the generation of specific components (107-109). Metabolic shift between anabolic and catabolic pathways is finely regulated and coupled to immunogenic or tolerogenic properties of DCs (108, 110, 111). When DCs are in a resting state, they exhibit low biosynthesis of macromolecules and highly efficient ATP production via fully active TCA cycle and OXPHOS. Fatty acid oxidation and glutaminolysis also provide intermediaries that enter the TCA cycle to fuel the synthesis of ATP (107, 109).

After activation, DCs shift their metabolism to a more rapid ATP generation by enhancing glycolysis and lactic fermentation despite the availability of oxygen, in a process called 'Warburg effect' (108, 109, 112). In addition, glycolytic intermediates are redirected through the PPP to generate key products for anabolic pathways (107). The TCA cycle also breaks allowing the generation of intermediary metabolites for fatty acid synthesis and molecules involved in the regulation of inflammatory responses, cytokine production and epigenetic modifications (17, 107, 113). This metabolic rewiring is mainly

driven by mammalian target of rapamycin (mTOR) signalling pathway, which activation drives anabolic processes (107, 114).

In contrast, tolerogenic DCs show an enhanced catabolic metabolism characterized by an increase OXPHOS and fatty acid oxidation (80, 81, 107, 115, 116). However, under certain conditions, tolerogenic DCs might also display a higher glycolysis with lactic acid fermentation. This process has an important relevance within the TME where lactate accumulation produced by tolerogenic DCs might supress proliferation of effector T cells and promote Treg cell generation (117).

2.3. Metabolic pathways in trained immunity

Training of innate immune cells is linked to profound changes in cellular metabolic pathways coupling the energetic need to the functional and epigenetic reprogramming changes (118, 119). Trained cells display a high glucose consumption with concomitant lactate production leading to enhanced ATP production, depending on the protein kinase B (Akt)/mTOR/hypoxia-inducible factor-1α (HIF-1α) axis (120-122). It has been shown that β-glucan-trained monocytes display a high glycolysis rate and Warburg effect with decreased OXPHOS (120). Remarkably, Arts et al. reported that BCG training also induces increased glucose consumption and OXPHOS in human monocytes, suggesting that both metabolic pathways can be activated during training (9, 122). In addition to pyruvate-derived acetyl-CoA, glutamine is also crucial to fuel the TCA cycle thought its direct conversion into the intermediate α-ketoglutarate from glutamine (9, 106). As a consequence of this shift of cellular metabolism towards TCA cycle, an accumulation of intermediate metabolites of the TCA cycle such as citrate, fumarate and succinate are produced (118, 121, 122).

Fumarate, succinate, citrate, acetyl-CoA or α-ketoglutarate, among others, regulate epigenetic modifications of histones and functional immune responses, which is crucial for the induction of trained immunity (17, 118). In addition, other metabolite derived from TCA cycle is itaconate, which acts as a modulator of inflammatory response, has an important role in the regulation of immune tolerance and training (123). Acetyl-CoA can also enter the cholesterol biosynthesis pathway during training induction and mevalonate pathway is also central for trained immunity, with a subsequent activation of mTOR pathway (124).

Compelling evidence indicates that the interplay between the metabolic and epigenetic rewiring is fundamental for the induction, maintenance, and regulation of cell functionalities (17, 125).

3. Epigenetics

3.1. An overview of the main epigenetic modifications

Epigenetics is defined as a stably heritable phenotype resulting from changes in the chromosome structure and accessibility for transcription without altering the underlying DNA sequences (126). The stimulation of immune cells leads to the upregulation of gene transcription of different cytokines and chemokines as well as the silencing of other non-necessary genes for the development of proper immune responses. There are multiple epigenetic mechanisms that tightly regulate gene transcription activation or repression such as DNA methylation, histone modification or noncoding RNA such as micro-RNA (127).

Histone modifications change chromatin structure and accessibility upregulating or downregulating the expression of specific genes. These modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation and deimination among others (128, 129). Histone acetylation are generally associated with gene transcription activation such as histone 3 acetylation of lysine 27 (H3K27ac), while trimethylations can mark active or repressive genes depending on the position such as histone 3 trimethylation of lysine 4 (H3K4me3) or histone 3 trimethylation of lysine 27 (H3K27me3), respectively (**Table 2**). Enrichments of histone modifications on specific genes can be analysed by chromatin immunoprecipitation (ChIP) assays. Histone modifications are generally localized within specific promoters or enhancers. For example, promoters are marked by H3K4me3 or H3K27me3, while enhancers are associated with histone 3 methylation of lysine 4 (H3K4me1). However, some histone modifications can mark both of them such as H3K27ac (130). Some groups of enzymes that modify histones are histone acetyltransferase, histone deacetylases, histone lysine methyltransferase or histone lysine demethylases (129).

Table 2. List of the main histone modifications.

Modification and position	Abbreviation	Effect on transcription	Location
Histone 3 lysine 27 acetylation	H3K27ac	Activating	Promoters, enhancers
Histone 3 lysine 4 methylation	H3K4me1	Activating	Enhancers
Histone 3 lysine 4 trimethylation	H3K4me3	Activating	Promoters
Histone 3 lysine 9 trimethylation	H3K9me3	Repressive	Broadly distributed
Histone 3 lysine 27 trimethylation	H3K27me3	Repressive	Promoters

Adapted from reference (130).

3.2. Epigenetic modifications in trained immunity

The epigenetic rewiring that drives the patterns of gene expression in the induction of trained immunity has been well studied. Some histone modifications associated with open active chromatin such as H3K4me1, H3K4me3 and H3K27ac mark promoters or enhancers of the pro-inflammatory genes *TNF*, *IL6* and *IL1B* on trained monocytes, thus increasing cytokine release after secondary stimulation (4, 9, 18). In contrast, the acquisition of the repressive histone 3 trimethylation of lysine 9 (H3K9me3) histone mark on the promoter of those cytokines suppress trained responses (9, 18, 131). However, epigenetic changes associated with training not only are involved in cytokine expression-related genes. Arts et al. has described that trained with BCG upregulate glycolysis-related genes by acquisition of H3K4me3 histone mark, thus decreasing the repressive H3K9me3 mark (122).

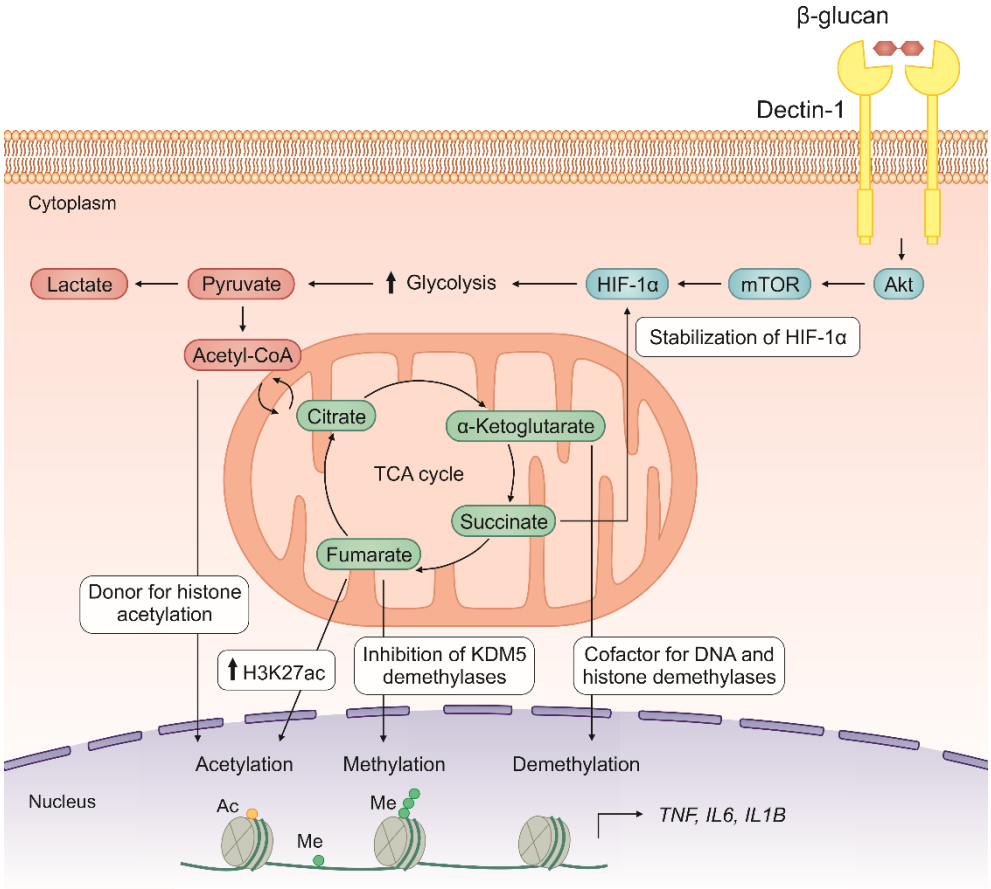

Figure 9. Main function of tricarboxylic acid (TCA) cycle intermediates in metabolic and epigenetic reprogramming. Ac, acetylation; Akt, protein kinase B; H3K27ac, histone 3 acetylation of lysine 27; HIF-1α, hypoxia-inducible factor-1α; Me, methylation; mTOR, mammalian target of rapamycin. Adapted from *Trained immunity: adaptation within innate immune mechanisms. Physiol Rev, 2023; 103(1):313-346.*

Metabolites derived from TCA cycle act as substrates or cofactors of enzymes involved in epigenetic changes (9, 17). Therefore, metabolites such as acetyl-CoA,

fumarate, succinate, citrate or α-ketoglutarate directly contribute to modulate the epigenetic reprogramming by mechanisms that activate or suppress enzyme activity (**Figure 9**). Acetyl-coA derived from pyruvate or citrate acts as donor for histone acetylation promoting pro-inflammatory gene expression (17). Fumarate increases H3K37ac and inhibits KDM5 demethylases, which promotes the trimethylation of H3K4me3 (121). Accumulation of succinate prevents the degradation of the transcription factor HIF-1α, which is involved in training (9). α-ketoglutarate is the cofactor for histone and DNA demethylases (9, 17).

4. Recurrent infections: genitourinary tract infections

Genitourinary tract infections (GUTIs), including urinary tract infections (UTIs) and vulvovaginal candidiasis (VVCs), represent a major health-care problem of increasing prevalence worldwide with a high socio-economic impact (132, 133). Although the most GUTIs are cleared up without complications, these infections affect the quality of life of patients, especially of those suffering from recurrences (133, 134). Antibiotics and antifungals remain the mainstay of treatment for these infections, respectively. However, their widespread use increases antimicrobial resistances and microbiota deleterious effects (133, 135-138).

4.1. Urinary tract infections (UTIs)

UTIs are one of the most common bacterial infectious diseases affecting more than 150 million people annually worldwide (132, 139). Approximately, 50% of women will experience at least one episode of UTI during their lifetime, and despite antibiotic therapy, 20-30% of women will develop recurrence (134, 140). Recurrent UTIs (RUTIs) are defined as two or more symptomatic UTIs within six months, or three or more symptomatic UTIs within a year (134, 141). For years, UTIs are broadly classified in uncomplicated, when patients have no risk factors for being susceptible to develop UTIs, and complicated UTIs, for patients with some risk factors (139, 142). Most uncomplicated UTIs are caused by *Escherichia coli* (around 80% of infections), followed by *Enterococcus faecalis*, *Staphylococcus saprophyticus*, *Klebsiella pneumonia* and *Proteus mirabilis* (142, 143).

Antibiotics are the mainstay of treatment for RUTIs, but the widespread long-term use increases antibiotic resistances, limiting current and future therapy efficacy specially for recurrent infections (144-146). In 2021, World Health Organization (WHO) has declare that antimicrobial resistance is one of the top 10 global public health threats that humanity will face in the near future (147). Remarkably, 1.27 million people died because

of antibiotic-resistant infections in 2019 before SARS-Cov-2 pandemic (148). In addition, other relevant adverse event associated with antibiotic overuse is the significant negative impact in the gastrointestinal and vagina microbiota, which subsequently might favor other bacterial and fungal secondary infections (135, 137, 149). Therefore, alternative strategies able to overcome these deleterious effects while preserving effectiveness are highly necessary (143, 150, 151). Among them, bacterial vaccines containing soluble antigens or inactivated whole bacteria, molecules impairing bacterial adhesion or probiotics are being studied as strategies for prevention and treatment of UTIs (150-154).

4.2. Vulvovaginal candidiasis (VVCs)

VVCs are vaginal infections primarily caused by *Candida* spp. More than 70% of women worldwide have at least once episode during their reproductive age (133, 155), and around 5-9% of women will experience a recurrence (155-157). VVCs are considered recurrent (RVVCs) when four or more episodes take place within a year (155). RVVCs affect more than 130 million women all over the world, impairing their quality of life (133). *C. albicans* remains responsible for around 90% of episodes of VVC, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* (155, 156, 158, 159). Among the predisposing factors described for the VVCs, it has been reported that antibiotics use increases 10-30% the risk to develop VVCs (137, 160, 161). After antibiotic exposure, protective vaginal bacteria such as *Lactobacillus* spp. are reduced which facilitate *C. albicans* proliferation and overgrowth. It has been proposed that this protective role of *Lactobacillus* might be due to the production of antimicrobial molecules such as lactic acid or hydrogen peroxide (H_2O_2) as well as features that impair the adherence to the epithelium (137, 162, 163).

The main recommended treatment for VVCs is topical or oral antifungals and among them, azoles are the most frequently prescribed, according to American and European Guidelines (164, 165). For RVVCs, long-term antifungal regimen with fluconazole is needed to prevent subsequent infections (164, 165). However, relapses are common when finishing the treatment, which require the long-term administration of antifungals again (166). Consequently, antifungal resistances are also emerging, thus becoming an additional health problem because of the extensive use of antimycotic drugs (136). Supporting this observation, high azole resistance rates for *C. albicans* has been described in women with VVCs (138, 167). Consequently, to overcome resistance to antifungals, new therapeutic strategies and other approaches are currently being evaluated (168-171).

4.3. Trained immunity-based vaccines (TIbV) as alternative therapeutic approaches

Antibacterial and antifungal vaccination strategies have been regarded as useful approaches to prevent and treat recurrent infections by boosting immune system (150, 169). Nowadays, vaccine development relies on next generation formulations that include non-specific immunomodulators able to potentiate and regulate proper immune responses against infections (172, 173). The traditional vaccines promote specific adaptive immune responses inducing antigen-specific memory T and B cells (174). Other vaccine preparations are also able to activate innate immune system promoting trained immunity (173). This has opened a new avenue in modern vaccinology through the development of trained immunity-based vaccines (TIbV) (20, 173, 175). TIbV are vaccine formulations that induce training in innate immune cells, thus stimulating broader responses against both related and unrelated antigens contained in the preparation (173, 176). These vaccines confer protection beyond the target infections exhibiting heterologous effects by mechanisms depending on enhanced innate and adaptive immune responses (**Figure 10**) (11, 20, 173).

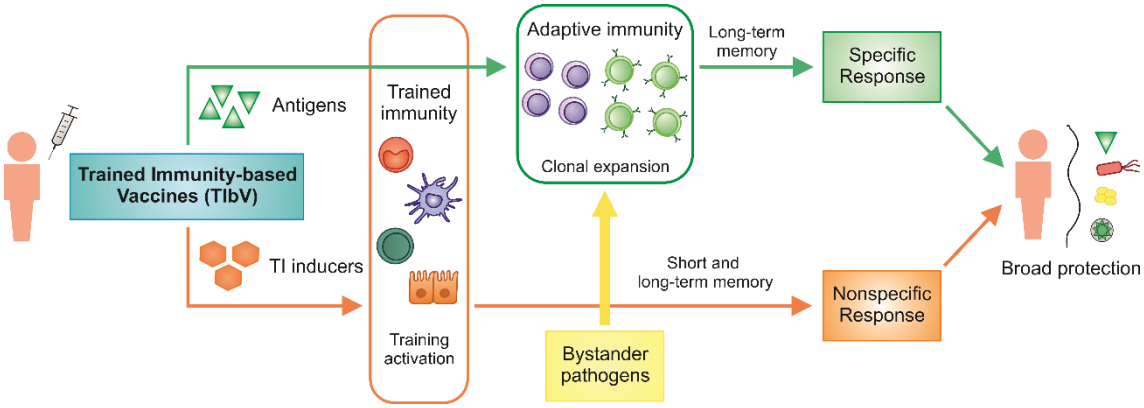

Figure 10. Mechanisms of trained immunity-based vaccines (TIbV) for infections. TIbV are formed by two essential components: trained immunity (TI) inducers and TIbV-related antigens. TI inducers reprogram innate immune, epithelial and hematopoietic stem cells so that they can respond in a much more efficient manner against bystander pathogens (both related and unrelated to those contained the formulation), thus resulting in a short/long-lasting memory. Under trained immunity conditions, more potent adaptive responses are orchestrated by T and B cells against both the antigens contained in the vaccine formulation but also against antigens contained in encountered bystander pathogens. TIbV are able to confer broad protection against both related and unrelated bystander pathogens. Adapted from *From trained immunity in allergy to trained immunity-based allergen vaccines. Clin Exp Allergy, 2022;00:1-11*.

The most studied TIbV is live attenuated *Mycobacterium bovis* BCG vaccine, which induces specific T cell responses and non-specific innate immune responses (11, 18). In addition, BCG promotes the training of bone marrow hematopoietic progenitors

involved in the long-lasting protection (15). BCG vaccination confers protection against heterologous infections, including *Staphylococcus aureus, C. albicans* and viruses (5, 12, 177). Others vaccines containing live attenuated or inactivated pathogens, such as measles vaccines, oral polio, vaccinia virus, influenza vaccines and MV130 vaccine for recurrent respiratory tract infections, have been associated with heterologous protection by trained immunity mechanisms (19, 178-182). Therefore, these vaccines represent a revolutionary therapeutic approach to reduce antimicrobial resistances by decreasing the use of antibiotics and antifungals, as well as to treat recurrent infections and heterologous pathogen-associated infections (172, 173, 183).

4.3.1. Mucosal vaccines

Although several injectable vaccines are highly effective, mucosal vaccines have gained a lot of attention over the last years. Mucosal vaccines elicit protective mucosal immune responses acting on first sites of pathogen contact (184, 185). Mucosal sites cover gastrointestinal tract, respiratory tract, urogenital tract and ocular sites, representing the predominant barrier of pathogen entry (184). The mucosa-associated lymphoid tissues (MALT) is a complex network of a highly specialized innate and adaptive mucosal immune cells and molecules involved in protecting from potential pathogen invasion as well as in maintaining homeostasis against innocuous antigens (185). MALT includes: gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT) and oral-associate lymphoid tissue (185). Although mucosal immune responses are compartmentalized, different mucosal sites are interconnected. In this regard, mucosal vaccination induces strong immune responses in local and peripheral lymphoid organs as well as in distant mucosal sites (184, 186). Mucosal tissues contain a large network of immune cells including APCs (DCs, B cells, macrophages and monocytes), which are recruited after pathogen exposure, and contribute to enhanced adaptive immune responses activating $CD4^+$ and $CD8^+$ T cells and inducing antigen-specific IgA (**Figure 11**) (184). Furthermore, the mucosal immune system also displays the capacity to generate tolerance against the microbiota, innocuous substances and self-antigens, as well as homeostasis to prevent harmful excessive immune responses after pathogen interactions (185).

Taken together, these observations explain why mucosal administration represents a suitable strategy to design vaccines with immunomodulatory capacity (19, 183, 185, 187, 188). Among them, sublingual vaccines have gained a lot of attention over the last years as a successful delivery route in allergen immunotherapy for allergic diseases and in vaccination for infectious diseases (99, 152, 187, 189). Sublingual

vaccination induces strong systemic immune responses and broad activation in distant mucosal sites. In contrast to oral immunization, this route avoids the degradation of antigens contained in the formulation in the gastrointestinal tract. Furthermore, there are no risk that antigens sublingually administered cross the blood-brain barrier, a weakness of intranasal route (189).

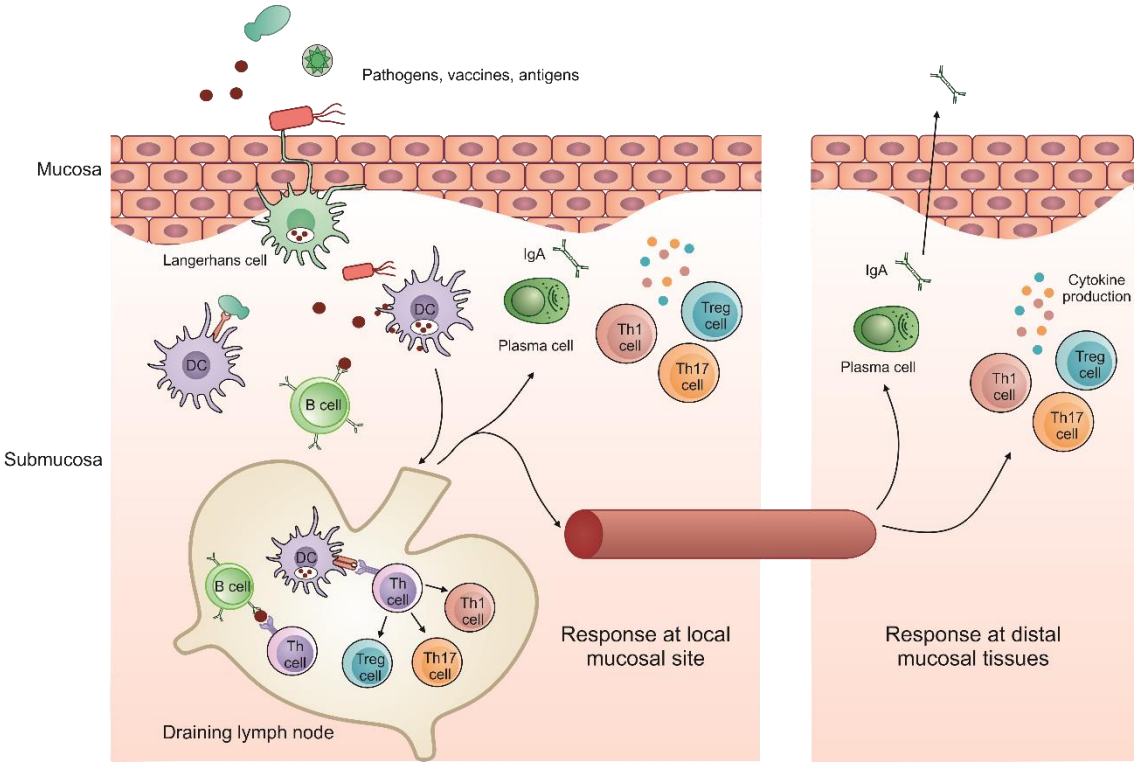

Figure 11. Immunologic mechanisms of sublingual mucosa. Pathogens, vaccines or antigens bind the epithelial cells and cross the mucosa, where they are uptake and processed by dendritic cells (DCs). DCs migrate to draining lymph nodes and present the processed antigens to naïve $CD4^+$ T cells to support the polarization into effector T and/or regulatory T (Treg) cells. B cells are activated and differentiated into IgA-producing plasma cells. Effector T cells and plasma cells back to the local mucosal tissues or migrate to distant mucosal sites to orchestrate immune responses. Adapted from *Update on immune mechanisms associated with sublingual immunotherapy: practical implications for the clinician. J Allergy Clin Immunol Pract, 2013; 1(3):228-41.*

4.3.2. Bacterial vaccine for RUTIs: MV140

The sublingual route is effective at inducing mucosal responses in urinary tract (152, 187, 189). Currently marketed mucosal vaccines for RUTIs contain bacterial soluble antigens (bacterial components or lysates) or inactivated bacteria for one or more species or strains (190). OM-89 (Uro-Vaxom®) is an oral preparation made up of bacterial lysates from 18 uropathogenic *E. coli* strains that significantly reduced the number of UTIs (153, 190). Regarding the use of inactivated whole pathogens, Urovac® is vaginal vaccine comprised of heat-inactivated six *E. coli* strains, *P. vulgaris*,

Morganella morganii, *E. faecalis* and *K. pneumonia*, that has been reported to reduce recurrent infections (190).

On the other hand, MV140 (Uromune®) from Inmunotek S.L. is a sublingual polyvalent bacterial preparation containing whole heat-inactivated bacteria causing the majority of RUTIs in Europe: 75% Gram-negative bacteria (25% *E. coli*, 25% *P. vulgaris* and 25% *K. pneumoniae*) and 25% Gram-positive bacteria (*E. faecalis*) (142, 191). Several clinical trials have demonstrated MV140 sublingually vaccination significantly reduced the number of UTIs in women (152, 192-196) compared with conventional antibiotics (195, 196). Two retrospective observational studies have recently reported a significant reduction in the number of episodes of RUTIs within six months and one year after the treatment (193, 194). Moreover, MV140 was also highly effective to reduce the rate of infections in men (193, 197). Interestingly, a significant decrease in the number of infections in elderly patients as well as patients with autoimmune diseases with recurrent RUTI, has been published in the last years (197, 198), showing that MV140 is a safe and efficacy vaccine in population at risk. Recently, a prospective study during two follow-up years in a single centre in Spain has reported a reduction in the consultation numbers in primary care and hospitals, diagnostic tests and antibiotic consumption in women with RUTIs treated with MV140, thus reducing the sanitary cost (199).

Mechanistically, MV140 acts on human DCs through CLRs and TLRs, promoting their maturation and the activation of NF-κB, p-38, extracellular-signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (78). Moreover, MV140 stimulation promotes the production of pro-inflammatory cytokines IL-1β, IL-6, IL-23 and TNFα and the anti-inflammatory cytokine IL-10 in DCs. MV140 endorses DCs with the ability to polarize Th1/Th17 and IL-10-producing T cells by mechanisms depending on Syk- and myeloid differentiation primary response 88 (MyD88)-mediated signalling pathways (**Figure 12**) (78). In addition, BALB/c mice sublingually immunized with MV140 display potent systemic Th1, Th17 and IL-10 responses (78). Although the beneficial use of MV140 has been demonstrated as a useful alternative to antibiotics, the role of this polybacterial preparation have never been explored in other infectious diseases. Due to the association of antibiotic therapy with the development of *C. albicans* infection, MV140 vaccination may contribute to prevent of VVCs.

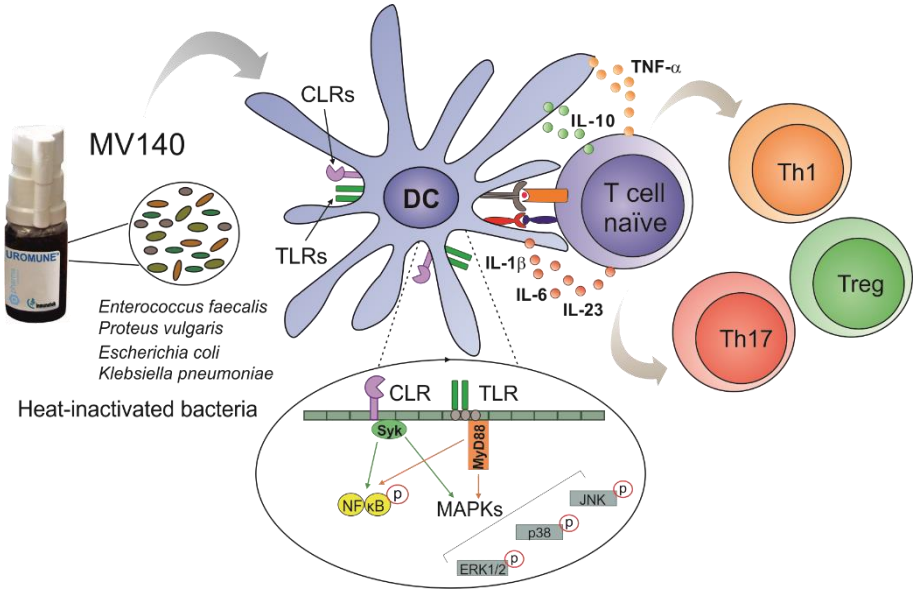

Figure 12. Molecular mechanisms involved in the mode of action of MV140. MV140 is a sublingual polybacterial preparation to prevent and treat recurrent urinary tract infections. MV140 acts on human dendritic cells (DCs) through C-type lectin receptors (CLRs) and Toll-like receptors (TLRs) by mechanisms depending on spleen tyrosine kinase (Syk) and myeloid differentiation primary response 88 (MyD88), which activates nuclear factor κB (NF-κB) and mitogen activated protein kinases (MAPKs) signalling pathways. MV140-activated DCs polarize Th1, Th17 and regulatory T (Treg) cell responses. ERK1/2, extracellular-signal regulated kinase 1/2; JNK, c-Jun N-terminal Kinase. Adapted from *Immunología. Revista oficial de la Sociedad Española de Inmunología (SEI). 2017; (36)1:23-24.*

4.3.3. Fungal vaccine for RVVCs: V132

Despite the efforts to develop strategies to control fungal infections and to reduce antimycotic resistances, effective fungal vaccines are not available (169, 200). Therefore, alternative therapeutics relying on vaccination might well represent a promising alternative to antifungals for the treatment of VVCs, as well as for prevention of recurrences. Different anti-*Candida* vaccine strategies have used live attenuated pathogens, recombinant surface or adhesion proteins, cell wall extracts or glycoconjugates (169, 200). Most of these approaches show interesting but still preliminary results. Among them, two recombinant vaccines have been assessed in human clinical trials (169). PEV7 is a recombinant secreted protein of *C. albicans* assembled into virosomes, but it has been only tested in healthy female intramuscular and intravaginal vaccinated (169, 201). On the other hand, NDV-3A contains a cell surface adhesion and invasin in the presence of aluminium hydroxide as adjuvant (169, 170). Intramuscular vaccination with NDV-3A was assessed in a clinical trial with women with RVVCs showing promising results (170).

Vaccine preparations based on whole pathogens contain several antigens and regulatory components that may induce enhanced immune responses compared to

recombinant vaccines formulated with a single antigen. As previously mentioned, sublingual bacterial vaccines formulated with whole heat-inactivated bacteria are successful for recurrent infections, suggesting that this approach may be useful for fungal infections. However, it has not been reported yet any heat-inactivated *C. albicans* vaccine assessed for RVVC (169). In this regard, V132 is sublingual fungal preparation based on whole heat-inactivated *C. albicans*. V132 has been developed by Inmunotek S.L. as an alternative strategy for the prevention and treatment of RVVCs. However, the mode of action of V132 has not been explored yet.

Given the tight relationship between RUTIs and RVVCs and the increasing adverse effects of antibiotics and antifungals, an alternative therapeutic strategy might be a formulation combining MV140 and V132 for the prevention of both GUTIs, but the detailed molecular mechanisms underlying the potential mode of action of this combination remained fully elusive prior to this Thesis.

5. Cancer

5.1. A brief overview of the cancer pathology

Cancer or malignant tumor is a complex multifunctional pathology in which abnormal cells grow uncontrollably in any organ or tissue of the body (202). Cancer cells may proliferate beyond their normal borders to invade adjoining parts of the body and/or spreading to other organs and developing new tumor colonies, in a process called ‘metastasis’. Metastasis is the major cause of death from cancer (202, 203). According to estimations from the WHO in 2020, cancer is, depending on the country, the first or second main cause of death worldwide, accounting for an estimated 10 million cancer deaths and 19.3 million new cancer cases (204). It is expected that global cancer burden increases a 47% until 28.4 million cases in 2040 (204). Female breast cancer, lung, colorectal, prostate and stomach are the most common types of cancer (**Figure 13**). In men, the most frequently diagnosed cancer are lung (4.3%), prostate (14.1%), colorectal (10.6%) and stomach cancer (7.1%); while in women are breast (24.5%), colorectal (9.4%), lung (8.4%) and cervix uteri cancer (6.5%) (204). The first leading cause of death is lung cancer, following by colorectal, liver, stomach, female breast, esophagus and pancreas cancers (204).

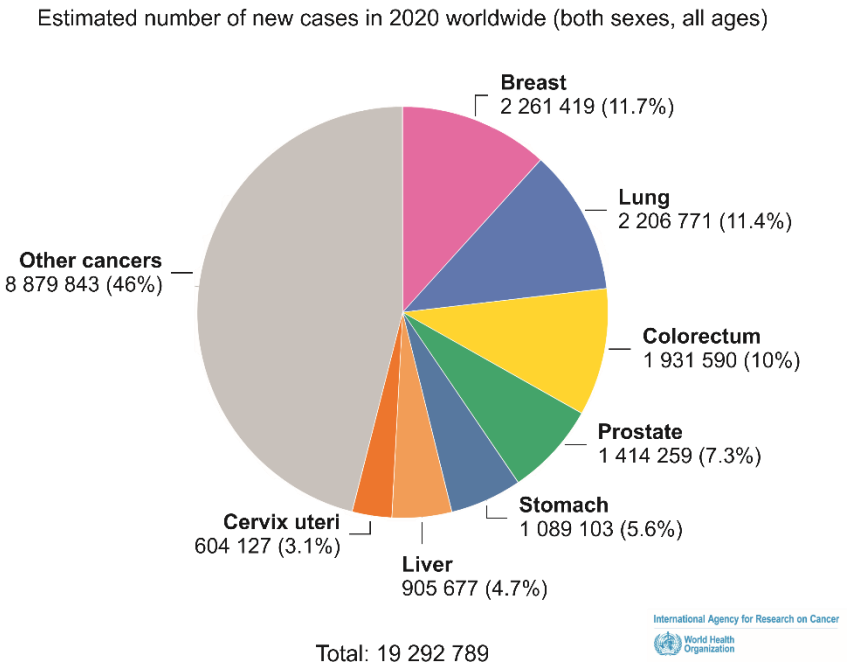

Figure 13. Estimated number of new cases in 2020 of different types of cancer in world population (both sexes, all ages). Adapted from *GLOBOCAN 2020 (http://gco.iarc.fr/).* Accessed December 12, 2022.

Hallmarks of cancer were proposed as a set of functional capabilities acquired by human cells during the malignant transformation, which are crucial for tumor development (205, 206). The fourteen hallmarks currently proposed include sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing or accessing vasculature, genome instability and mutation, resisting cell death, deregulating cellular metabolism, nonmutational epigenetic reprogramming, unlocking phenotypic plasticity, polymorphic microbiomes and senescent cells (205).

Currently, different therapeutic approaches for cancer patients depend on the type of tumor, site and state of cancer, among others. Conventional treatment strategies include surgery, chemotherapy and radiotherapy (207). Despite their effectiveness alone or combined for cancer treatment, they still have some important limitations. Surgery is the most effective therapy, but some tumor cells may not be completely removed in certain cases. Chemotherapy might kill in a non-specific manner healthy cells and in addition, cancer cells can acquire drug resistance. Radiotherapy can also damage healthy cells (207). In the last years, the development of new strategies has focused on a further inhibition of cancer proliferation and dissemination, while decreasing the damage of healthy cells (207). In this regard, immunotherapy enhances the immune system capacity to supress and eliminate tumor cells. Monoclonal antibodies (mAbs) are designed to kill cancer cells through different mechanisms including blocking signalling

pathways, enhancing antibody-dependent cell-mediated cytotoxic (ADCC), activation of complement system, immune checkpoint inhibition, and activation of costimulatory receptors (208, 209). Immune checkpoints, such as CTLA-4 and PD-1/PD-L1, prevent excessive immune responses in healthy conditions, as previously shown in **Figure 4** (208, 209). The first mAbs for cancer immunotherapy were developed against CTLA-4 (ipilimumab, tremelimumab), PD-1 (nivolumab, pembrolizumab) and-PD-L1 (atezolizumab, durvalumab) with successful efficacy (210). In addition, other mAbs for different checkpoints are being currently tested (208, 210).

Although the intensive performed research to develop novel cancer treatments is highly encouraging, a better understanding of the complex interplay between the immune system and cancer cells may lead to improved approaches in the field of immunotherapy.

5.2. Immune system in cancer

Compelling evidence demonstrated the involvement of the immune system in both tumor rejection and tumor progression (211-213). During the early stages of tumor development, immune cells including neutrophils, NK cells, macrophages, DCs, effector $CD8^+$ and $CD4^+$ T cells, are recruited to the neoplastic place and contribute to eliminate cancer cells (**Figure 14**) (211). **Neutrophils** rapidly arrive to tumor site and, as well as macrophages, display dual functions in the TME. In tumor initiation, neutrophils mediate the elimination of cancer cells through direct contact and via the generation of nitric oxide (NO). Interestingly, components of TME promote the maturation of neutrophils expressing MHC molecule and they can act as antigen-presenting cells (214). Anti-tumor **M1-like macrophages** mediate inflammation and cytotoxicity to kill cancer cells. Moreover, M1 macrophages release cytokines (IL-6, TNFα and IL-12) to activate other immune cells such as NK cells and $CD8^+$ T cells (215). **NK cells** can directly kill tumor cells through mechanisms depending on the release of perforin and granzyme and the induction of cancer cell apoptosis. Stimulatory receptors of NK cells such as NKG2D bind diverse products from cancer cells activating NK cell cytolytic activity. In healthy conditions, inhibitory receptors recognize MHC class I resulting in NK cell inhibition, whereas lacking of MHC class I in cancer cells activates NK cells (211). Tumor-infiltrating **DCs** and DCs from tumor-draining lymph nodes acquire tumor-associated antigens (TAAs) and present or cross-present them to T cells. The ability of DCs to promote pro-tumor effector T cells or highly suppressive Treg cells is strongly influenced by exogenous signals encounter in the TME (21). Tumor-infiltrating mDC1 produce C-X-C chemokine ligand (CXCL)9 and CXCL10 which recruit CTLs into tumor site for cross-presentation, and IL-12 which promote CTL and NK cell cytotoxicity as well as IFNγ

production (21, 74). mDC2 contribute priming $CD4^+$ T cells (66). In addition, pDCs exhibit an important anti-tumor capacity producing type I IFN, which activate CTL response (21, 74). During tumor progression, **monocytes** are recruited to tumor site for differentiation into DCs with immunogenic or tolerogenic features depending on the signals encountered in the TME (74, 75, 216).

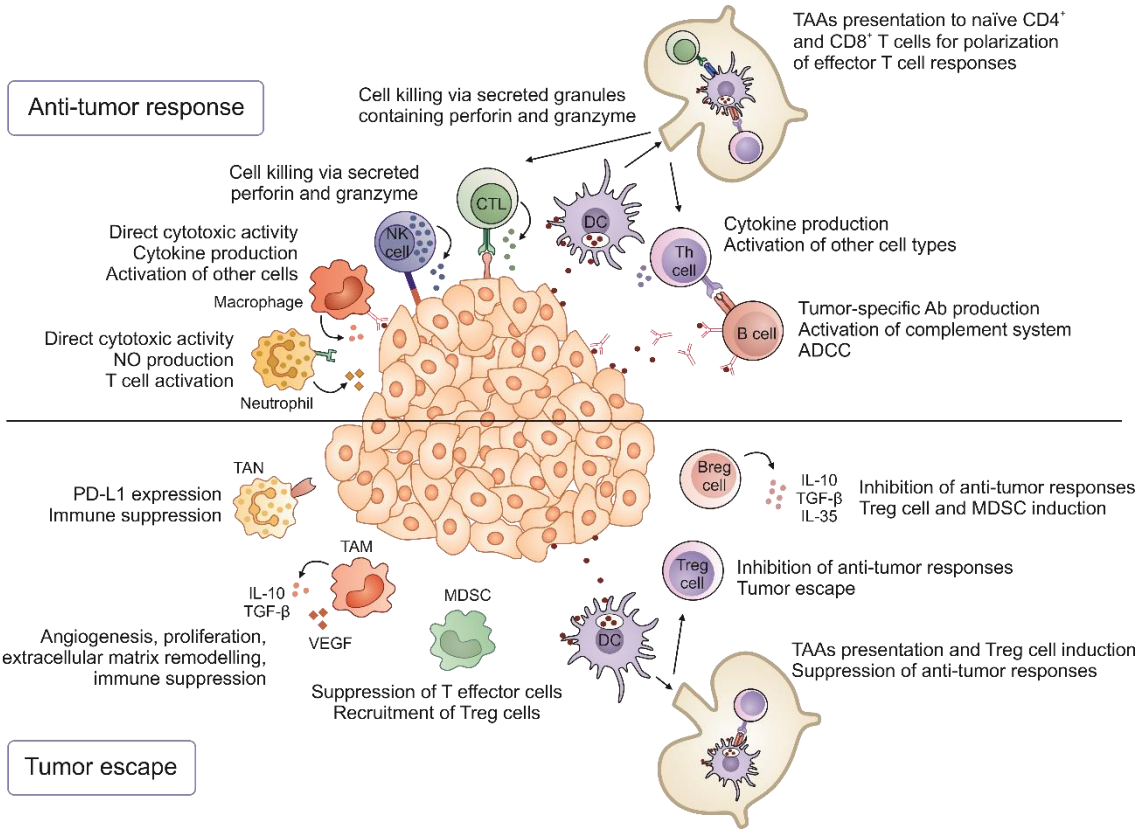

Figure 14. Main immune cells and mechanisms involved in anti-tumor responses and tumor escape. Neutrophils, pro-inflammatory M1 macrophages, natural killer (NK) cells, dendritic cells (DC), $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T cells and B cells lead an effective anti-tumor responses. Tumor-associated neutrophils (TAN), tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), DCs, regulatory T (Treg) cells and B regulatory (Breg) cells favour tumor progression and suppress the anti-tumor immune responses. ADCC, antibody-dependent cellular cytotoxicity; NO, nitric oxide; PD-L1, programmed cell death-ligand 1; TAAs, tumor-associated antigens; VEGF, vascular endothelial growth factor.

On the one hand, **CTLs** display a potent anti-cancer function secreting granules containing granzymes, perforin, cathepsin C and granulysin to promote the lysis of tumor cells (217). On the other hand, **Th1 cells** produce cytokines (IFNγ, TNFα and IL-2) that promote the recruitment of NK cells, macrophages and CTLs. **Th17 cells** produce IL-17, which display a dual role by polarizing M1-like macrophages and enhancing NK cell and CTL activity, but also myeloid-derived suppressor cells (MDSCs). It has been described that IL-4 produced by **Th2 cells** activated both NK cells (anti-tumor) and anti-inflammatory M2 macrophages (pro-tumor) (218). Furthermore, **B cells** produce tumor-

specific antibodies which are involved in phagocytosis by macrophages and DCs, complement activation and ADCC (**Figure 14**) (219).

As the tumor progress, TME changes resulting in a polarization of **tumor-associated macrophages (TAMs)** and **tumor-associated neutrophils (TANs)** takes place. TAMs are anti-inflammatory M2-like macrophages that display a pro-tumor phenotype. TAMs produce soluble factors that favour angiogenesis, cancer cell proliferation, remodelling extracellular matrix, metastases and immune suppression (IL-10 and TFG-β which impair DC maturation and effector T cells) (211, 215). TANs can express immune checkpoints such as PD-L1 driving T cell suppression (211, 214). **DCs** generate Treg cells in lymph nodes and TME, and produce by themselves inhibitor factors that impairs effector cell activity and chemokines such as CCL2 to induce Treg cell migration (21, 74, 220). In addition, different molecules such as IL-6, gangliosides and prostaglandine E2 (PGE2) inhibit DC recruitment, differentiation and maturation, and induce tolerogenic DCs (21, 74). Moreover, type I IFN production by pDCs is inhibited (21). **Treg cells** are recruited into tumor sites playing a key role in tumor progression as well as suppressing the immune response (98, 104). Recently, **MDSCs** have been identified as a heterogeneous population of immature myeloid cells with suppressive capacity. MDSCs inhibit anti-tumor immune response supressing T effector cells and recruiting Treg cells (221). Furthermore, expansion of **Breg cells** producing IL-10, IL-35 and TGF-β has been correlated with inhibition of CTLs, effector $CD4^+$ T cells, NK cells and monocytes, and induction of Treg cells and MDSCs (**Figure 14**) (222).

5.2.1. Treg cells in cancer immunology

Functional Treg cells are abnormally expanded in cancer and enriched in different tumor tissues having a highly suppressive capacity. They play a crucial role in tumor development and suppression by impairing proper host anti-tumor immune responses (104, 223-225). In tumor tissues, Treg cells can be generated from local $FOXP3^-$ conventional T cells and/or recruited to the tumor site upon previous expansion in lymphoid organs (98, 99, 104, 226).

Several studies have reported that Treg cells represent a major barrier to anti-cancer immunity thought multiple tightly regulated mechanisms (**Figure 15**) (104, 225, 227). Intratumoral Treg cells regulate tumor angiogenesis through their production of vascular endothelial growth factor (VEGF) (104). Intratumoral Treg cells display multiple immune checkpoints and costimulatory molecules that finely promote immune suppression as previously described (104, 225). Naïve $CD4^+$ T cells are differentiated into Treg cells in the presence of TGF-β, which can be available in the TME and/or

produced by Treg cells themselves (104, 228). In addition, Treg cells secrete other immunomodulatory molecules including IL-10 and IL-35, thus inhibiting effector T cells, NK cells and DCs (227, 229). Treg cells also produce cytotoxic molecules such as granzymes and perforines to lyse $CD4^+$ T cells, CTLs and APCs (104, 227). Effector T cells produce IL-2 that is necessary for survival and proliferation of Treg cells. Treg cells express high affinity IL-2 receptor complex (CD25, CD122 and CD132), which binds IL-2 and, consequently, IL-2 depravation impairs the function and expansion of effector T cells (225). CD39 and CD73 catalyse the conversion from ATP to ADP and adenosine. Adenosine binds adenosine receptor A2A on effector T cells and DCs suppressing their activity (104, 230). Moreover, Treg cells generate cAMP which is directly transferred to effector T cells inhibiting their function (227).

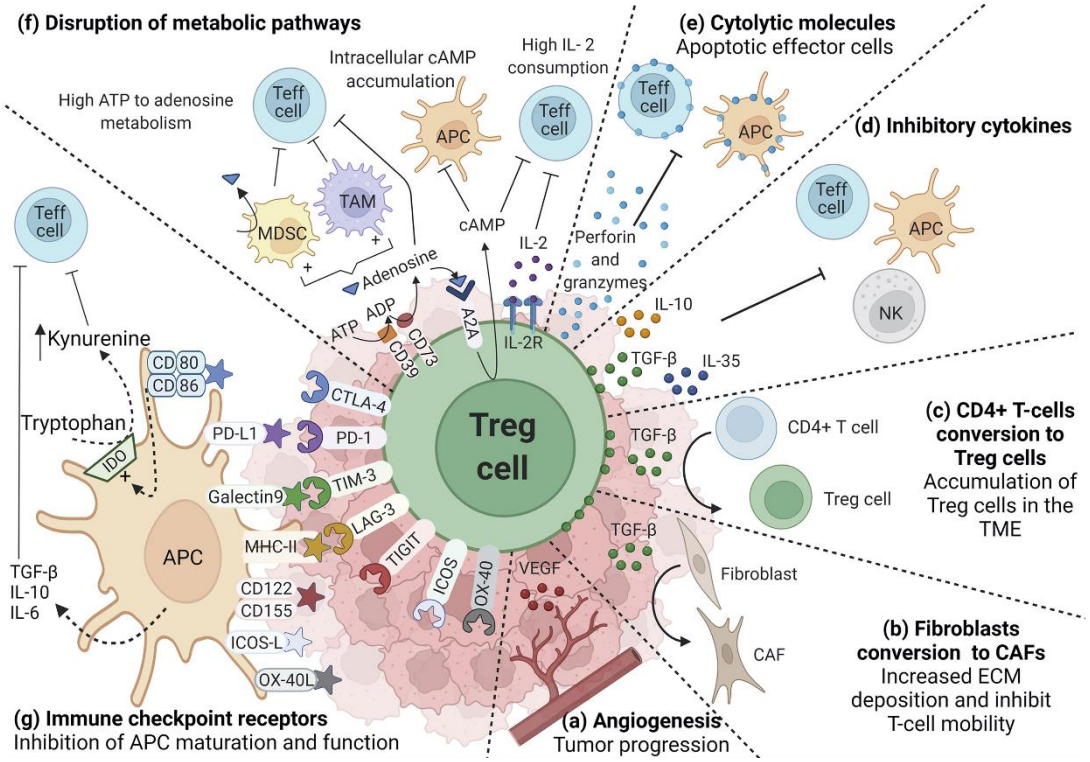

Figure 15. Tumor-infiltrating regulatory T (Treg) cells and mechanisms of suppression. Treg cells supress host anti-tumor immune responses by inhibiting different crucial events. A2A, adenosine A2A receptor; APC, antigen-presenting cells; CAFs, cancer-associated fibroblasts; cAMP, cyclic adenosine monophosphate; CTLA4, cytotoxic T lymphocyte antigen 4; ECM, extracellular matrix; ICOS, inducible T-cell costimulator; ICOSL, inducible costimulator ligand; IDO, indoleamine 2,3-dioxygenase; LAG3, lymphocyte activation gene 3; MDSC, myeloid-derived suppressor cells; MHC, major histocompatibility complex; NK, natural killer; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; TAM, tumor-associated macrophages; Teff, effector T; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domains; TIM-3, T cell immunoglobulin and mucin domain-containing 3; TME, tumor microenvironment; Treg, regulatory T; VEGF, vascular endothelial growth factor. Adapted from *Colorectal cancer-infiltrating regulatory T cells: functional heterogeneity, metabolic adaptation, and therapeutic targeting. Front Immunol, 2022; 13:903564*.

5.3. Tumor-associated carbohydrate antigens

Besides immunosuppression in TME, avoiding immune recognition or immunoediting are other mechanisms of tumor scape. Tumor evades the anti-tumor immune response using two main strategies: (i) losing the tumor antigens expression on the cell surface, and (ii) modifying these antigens to avoid the immune recognition. Malignant transformation is accompanied by changes in tumor cell surface glycoconjugates (231), including aberrant or incomplete glycosylation of heavily glycosylated structures such as mucins and proteoglycans (232). Consequently, new carbohydrate epitopes are generated called 'tumor-associated carbohydrate antigens' (TACAs) (233). These structures are often overexpressed and shed from tumor cells, having a strong influence on tumor progression by modulating cell adhesion, angiogenesis and metastases (234-236). Tumor-associated carbohydrates might inhibit anti-cancer immunity through different mechanisms (237-239) such as the expansion of MDSCs (240, 241) or the activation of Treg cells (236).

Mucins are composed of oligosaccharide chains covalently attached to proteins, whereas proteoglycans consist of a protein core covalently attached to one or more glycosaminoglycan chains such as heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid (234, 242). The attachment of glycans to proteins can be mediated by *N*-linked glycosylation (binding to the amine group of asparagine) or *O*-linked glycosylation (binding to the hydroxyl group of mainly serine or threonine). Aberrant glycosylation might involve an increase in *N*-glycan branching, *O*-glycan density or incomplete glycosylation (232, 243). Several studies have reported TACAs attached to different structures in a wide variety of cancers (232). Due to their overexpression in certain tumors, some of these TACAs can be employed in clinic for diagnosis, prognosis and/or monitoring such as carcinoembryonic antigen (CEA) for colon cancer, prostate specific antigen (PSA) for prostate cancer, Mucin 1 (MUC1 or CA15-3) for breast cancer, or sialyl-Tn antigen (sTn, CA72-4) for gastric cancer (243).

5.4. Carbohydrate A10 (Ca10)

The carbohydrate A10 (Ca10) is a highly glycosylated Ehrlich tumor (ET) cell surface macromolecule defined by the binding of the monoclonal antibody A10 (244, 245). Murine ET cells, originally derived from a spontaneous mammary carcinoma, lack of H-2 antigens and can grow either in ascitic or solid form in almost any mouse strain across different antigenic backgrounds (246, 247). The IgM antibody A10 inhibits ET growth both *in vitro* (248) and *in vivo* (244). Interestingly, immunized mice with devitalized ET cells showed a production of specific IgM antibodies that conferred resistance to tumor

establishment (249). In addition, it was reported an association between the presence of natural IgM antibodies against ET cells with resistance to tumor growth (244). Mechanistically, the antibody A10 might mediate tumor cytostasis promoting the adhesion between macrophages and ET cells (248). Ca10 is spontaneously released from ET cells and can be detected in the serum of solid ET-bearing mice (245).

The structural features of the carbohydrate fraction of Ca10 remained unknown. In ET, the carbohydrate Ca10 was initially described as a high molecular weight glycoprotein sensitive to sodium metaperiodate ($NaIO_4$) oxidation (245). Remarkably, the antibody A10 also reacts with glycan moieties located in some human cancer mucins (250). It has been suggested the presence of a circulating human Ca10 counterpart in patients with diverse types of cancer (unpublished data). However, the potential functional implications of the carbohydrate Ca10 in cancer and its structural features of the carbohydrate moiety remains largely unknown.

OBJECTIVES

Recurrent infections and cancer represent major healthcare problems of increasing prevalence with high socio-economic burden.

The widespread use of antibiotics and antifungals as the main treatments for bacterial and fungal infections, respectively, contributes to increase microbial resistances and to favor microbiota deleterious effects. The development of novel strategies to treat and prevent infections are, therefore, highly demanded. Among them, the bacterial (MV140) and fungal (V132) sublingual vaccines might well represent promising alternative therapies for genitourinary tract infections (GUTIs). The better understanding of their mode of action at the cellular and molecular level is needed.

Malignant transformation is accompanied by changes in tumor cell surface glycoconjugates generating new carbohydrate epitopes. These tumor-associated carbohydrate antigens mediate tumor progression and condition immune responses, thus promoting tolerance against the tumor. In this context, the carbohydrate A10 (Ca10), a cell-surface carbohydrate derived from Ehrlich tumor (ET) cells, might have functional implications in cancer development.

Dendritic cells (DCs) are essential for the generation and maintenance of proper immune responses by linking innate and adaptive immunity. DCs have been regarded as suitable therapeutic targets for immune-mediated diseases such as recurrent infections or cancer.

We hypothesized that MV140 and V132 formulations as well as the aberrant tumoral carbohydrate Ca10 could modulate the functional properties of human DCs, which might well contribute to the restoration of proper anti-infection immune responses or to tumoral progression, respectively. The better understanding of the molecular mechanisms by which MV140/V132 and the tumoral carbohydrate Ca10 regulate immune responses might well contribute to the development of alternative interventions for GUTIs and cancer.

Considering all these aspects, the main goal of this Doctoral Thesis is to study the underlying molecular mechanisms by which the bacterial (MV140) and fungal (V132) sublingual vaccines for recurrent GUTIs as well as the tumoral carbohydrate Ca10 from ET cells, regulate the functional properties of human DCs, including their ability to polarize T cell responses.

To achieve this main goal, we pursue the following specifics aims divided into two different blocks:

Block A: Study of the molecular mechanisms involved in the mode of action of the fungal vaccine V132 and the polybacterial preparation MV140 for recurrent genitourinary tract infections.

The main aim of this block is to study the immunomodulatory properties of V132, MV140 and the combination of both of them (MV140/V132) in human DCs, as well as their capacity to promote trained immunity in human PBMC and monocytes.

Objective 1. Study of the molecular mechanisms involved in the capacity of MV140/V132 to immunomodulate human DCs.

1.1. To study the capacity of MV140/V132 to regulate the phenotype and function of human DCs.
1.2. To study the polarization of T cell responses induced by human DCs stimulated with MV140/V132.
1.3. To study the main immune signalling pathways involved in the immunomodulatory effects of MV140/V132 in human DCs.
1.4. To study metabolic and epigenetic changes induced by MV140/V132 in human DCs.
1.5. To study the immunomodulatory properties of MV140/V132 in an *in vivo* mice model.

Objective 2. Assess the capacity of V132, MV140 and MV140/V132 to induce trained immunity in preclinical human and mice models and elucidation of the underlying molecular mechanisms.

2.1. To study the capacity of V132, MV140 and MV140/V132 to induce trained immunity in human PBMC.
2.2. To study the main metabolic and epigenetic changes promoted by V132 in human PBMC.
2.3. To study the capacity of V132, MV140 and MV140/V132 to induce trained immunity in human monocytes.
2.4. To study the capacity of V132 to induce trained immunity in an *in vivo* mice model.

Block B: Study of the molecular mechanisms by which the tumoral carbohydrate Ca10 immunomodulates human DCs.

The main aim of this block is to study the immunological mechanisms by which the tumoral carbohydrate Ca10 regulates the phenotype and function of DCs as well as their capacity to polarize T cell responses.

Objective 3. Study of the capacity of the tumoral carbohydrate Ca10 to induce tolerogenic human DCs and the elucidation of underlying molecular mechanisms.

3.1. To assess the potential relationship between circulating Ca10, tumor size and Treg cell generation in a solid Ehrlich tumor-bearing mice model.

3.2. To study the capacity of the tumoral carbohydrate Ca10 to regulate the phenotype and function of human DCs.

3.3. To study the main immune signalling pathways involved in the immunomodulatory effects imprinted by Ca10 in human DCs.

3.4. To study the metabolic changes and their contribution to the tolerogenic properties exerted by Ca10 in human DCs.

3.5. To study the capacity of the tumoral carbohydrate Ca10 to generate Treg cells and the underlying molecular mechanisms involved in such effects.

3.6. To assess the specific contribution of the carbohydrate moiety of Ca10 and the A10 epitope to condition the capacity of DCs to polarize T cell responses, with special focus on the generation of Treg cells.

Objective 4. Study of the capacity of the tumoral carbohydrate Ca10 to reprogram monocyte differentiation into tolerogenic DCs.

4.1. To study whether the tumoral carbohydrate Ca10 could reprogram human monocytes into tolerogenic DCs.

4.2. To study the capacity of hmoDCs differentiated in the presence of Ca10 to polarize Treg cells.

MATERIALS AND METHODS

1. Material, media and reagents

RPMI 1640 medium (Lonza) supplemented (cRPMI) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 µg/mL normocin (InvivoGen), 50 µg/mL penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mM sodium pyruvate (all from Life Technologies) was used for *in vitro* DC and T cell cultures, and for animal experiments.

RPMI culture medium Dutch modified (Invitrogen) supplemented with 50 µg/mL gentamicin, 2 mM Glutamax (Gibco) and 1 mM pyruvate (Gibco) was used for human *in vitro* trained immunity experiments.

We used lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 for *in vitro* DC cultures, and O155:B5 (Sigma-Adrich) for *in vitro* trained immunity culture and animal models. Inhibitors for histone methyltransferase (5′-deoxy-5′-methylthioadenosine, MTA), histone demethylase (Pargyline), IDO (1-methyl tryptophan, 1-MT), glycolysis (2-Deoxy-D-glucose, 2-DG), and NF-κB (BAY117082) (Sigma-Adrich); Syk (piceatannol) (Enzo Life Sciences); MEK 1/2 (U0126), p38 (SB202190), JNK (SP600125) and mTOR (Rapamycin) (InvivoGen) were employed. Heparin (Laboratorio Reig Jofré) and calcium chelating agent (ethylenediaminetetraacetic acid, EDTA) were used. Blocking antibodies for IL-10 (clone JES3-9D7), and PD-L1 (clone 29E.2A3, Biolegend) were employed. Corresponding vehicles or isotype controls were used for the blocking experiments.

2. Polybacterial and fungal preparations

V132, MV140 (Uromune®), MV140/V132 and control excipients were provided by Inmunotek S.L. V132 is composed of whole heat-inactivated *C. albicans*. MV140 (Uromune®) is a polybacterial preparation composed of whole heat-inactivated bacteria: 75% Gram-negative bacteria (25% *E. coli*, 25% *P. vulgaris* and 25% *K. pneumoniae*) and 25% Gram-positive bacteria (*E. faecalis*). MV140/V132 contains 50% MV140 and 50% V132 in a single preparation. Control excipients is identical for MV140 and V132 and contains all excipients without bacteria and yeast (glycerol, saline and pineapple flavour).

3. Carbohydrate A10 (Ca10)

Carbohydrate A10 (Ca10) was provided by Inmunotek S.L. Ca10 was obtained from supernatants of Ehrlich tumor (ET) cells cultured 24 hours at high cell numbers in serum-free medium. Briefly, the pooled supernatants collected by centrifugation were subjected to tangential ultrafiltration on 300 kDa membranes (Pall Corporation) under high ionic

strength (1 M NaCl). The Ca10-enriched fraction, which was completely recovered in the retentate, was subsequently diafiltrated against distilled water on 300 kDa membranes and lyophilized, having a final composition of 90% carbohydrate and 10% protein.

In some experiments, Ca10 was oxidized (Ca10 OX) with 50 mM sodium periodate (Sigma-Aldrich) during 70 minutes at room temperature in the dark. Distilled water was then added and centrifuged (14000*g*, 10 minutes) using Nanosep 3K Omega (Pall Corporation). Ca10 was subjected to enzymatic treatment with Bacteroides Heparinase II (85 AU/mL) plus Heparinase III (14.8 AU/mL) (New England Biolabs) prepared in Heparinase Reaction Buffer during 24 hours at 30ºC (HPSE treat.).

Monoclonal IgM antibody A10 was provided by Inmunotek S.L. A10-producing hybridomas were previously obtained by immunization with devitalized ET cells and selected due to their reactivity against carbohydrates on the surface of murine ET cells as well as their ability to inhibit *in vivo* tumor growth (244). Briefly, monoclonal antibody A10 was obtained from supernatants of A10-producing hybridoma after 72 hours of *in vitro* culture. The pooled supernatants collected by centrifugation were subjected to chromatography affinity purification (HiTrap IgM Purificación HP, GE Healthcare) specific to retain IgM. Finally, the purified monoclonal antibody A10 was dialyzed and the reactivity against ET cells and the tumoral carbohydrate Ca10 was evaluated.

4. Isolation of different human cell subsets

4.1. Isolation of peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors (Transfusion Centre, Madrid, Spain) by Ficoll-Paque (GE Healthcare) density gradient centrifugation (800*g*, 20 minutes) at room temperature. The interphase layer containing PBMC was collected and cells were washed with PBS and re-suspended in cRPMI medium. Cell viability was analysed using Trypan blue in a Neubauer chamber under a light microscope.

4.2. Purification of monocytes

Human blood monocytes were isolated from total PBMC using anti-CD14 microbeads (Miltenyi Biotec) and the autoMACS Pro (Miltenyi Biotec) through positive selection according to manufacturer's instructions. Cell viability was analysed using Trypan blue in a Neubauer chamber under a light microscope.

4.3. Generation of human monocytes-derived DCs (hmoDCs)

Purified monocytes were differentiated into immature human monocytes-derived DCs (hmoDCs). Monocytes were cultured in 24-well plate at a concentration of 10^6 cells/mL with cRPMI medium in the presence of 100 ng/mL IL-4 and GM-CSF (PeproTech) for 6 days at 37ºC in a humidified 5% CO_2 incubator. To generate hmoDCs differentiated in the presence of Ca10 (Ca10/hmoDCs), 20 µg/mL of Ca10 was added at days 0 and 4 of the differentiation (**Figure 16**). The purity and phenotype of monocytes and hmoDCs were analysed by flow cytometry with lineage-specific markers such as HLA-DR, CD83, CD86 or CD14 at Centro de Citometría de Flujo y Microscopía de Fluorescencia (CCMF) from Complutense University of Madrid (UCM).

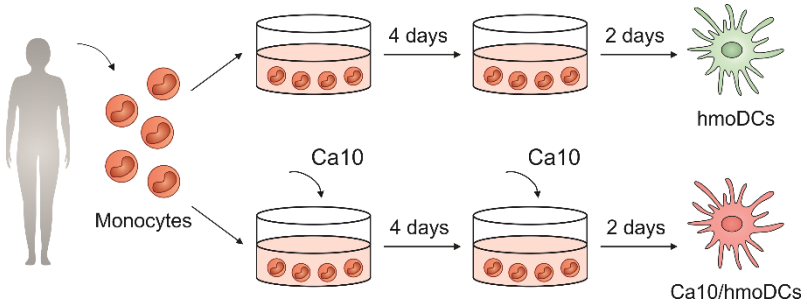

Figure 16. Human *in vitro* model for the generation of conventional hmoDCs and Ca10/hmoDCs.

4.4. Purification of naïve $CD4^+$ T cells

Naïve $CD4^+$ T cells were purified from PBMC using the 'Naïve $CD4^+$ T Cell Isolation Kit' (Miltenyi Biotec) and the autoMACS Pro (Miltenyi Biotec) according to manufacturer's protocol with minor modifications. The cells were purified through a negative selection labelling all the rest of the cells using microbeads conjugated to following antibodies: CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR and CD235a antibodies. The purity of the obtained naïve $CD4^+$ T cells was assessed by flow cytometry with anti-CD45RA, anti-CD4 and anti-CD3 antibodies. Cell viability was analysed using Trypan blue in a Neubauer chamber under a light microscope.

4.5. Purification of total blood DCs

Total DC fraction was obtained from PBMC using the 'Blood Dendritic Cell Isolation Kit II' (Miltenyi Biotec) and the autoMACS Pro (Miltenyi Biotec) following manufacturer's instructions with minor modifications. The purity of the enriched total DC fraction contained both pDCs and mDCs was analysed by flow cytometry with anti-CD1c, anti-CD303, anti-CD19 and anti-HLA-DR antibodies. Cell viability was analysed using Trypan blue in a Neubauer chamber under a light microscope.

4.6. Isolation of PBMC and monocytes for trained immunity experiments

Human PBMC were obtained from buffy coats of healthy donors (Sanquin Blood Bank, Nijmegen, the Netherlands) by Ficoll-Paque (GE Healthcare) density gradient centrifugation (615*g*, 30 minutes) at room temperature. The interphase layer containing PBMC was collected and cells were washed with PBS, re-suspended in RPMI culture medium Dutch modified and counted (251). Viability was analysed using the Sysmex XN-450.

Monocyte isolation from PBMC was performed by hyper-osmotic Percoll (Sigma-Aldrich) density gradient centrifugation (580*g*, 15 minutes) at room temperature. The interphase layer was collected, and cells were washed with cold PBS, re-suspended in RPMI culture medium Dutch modified and counted (251). Viability was analysed using the Sysmex XN-450. An extra purification step was added by adhering Percoll-isolated monocytes or Ficoll-isolated PBMC to a flat-bottom 96-well plates for 1 hour at 37ºC. Subsequently, monocytes were washed with warm PBS to yield maximal purity.

5. Cell culture procedures

5.1. Culture of human DCs

Immature hmoDCs from healthy donors (10^6 cells/mL) were stimulated with control excipients (negative control), V132 (3 FTU, Formazan Turbidity Units, per mL), MV140 (10^7 bact. per mL) or MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL) for 18 hours at 37ºC in a humidified 5% CO_2 incubator. Cell pellets were used to assess their phenotype by flow cytometry using the following markers: HLA-DR, CD83 and CD86; and for the analysis of the mRNA levels of *COXII*, *GAPDH* and *HIF1α* by qPCR. Cell-free supernatants were used to quantify IL-6, TNFα, IL-1β, IL-23, IL-10 and TGFβ1 by sandwich enzyme-linked immunosorbent assay (ELISA). For inhibition experiments, hmoDCs were pre-incubated for 1 hour with MTA (0.5 mM) or Pargyline (3 mM) (or their vehicle control, DMSO) prior to activation. Then, cells were stimulated with stimuli for 18 hours in the presence of the corresponding inhibitors to quantify TNFα, IL-23 and IL-10 by ELISA.

Immature hmoDCs or human total blood DC fraction from healthy donors (10^6 cells/mL) were stimulated with medium (negative control) or Ca10 (20 µg/mL) for 18 hours at 37ºC in a humidified 5% CO_2 incubator. Cell pellets were used to assess their phenotype by flow cytometry using the following markers: HLA-DR, CD86, CD83 and PD-L1; and for the analysis of the mRNA levels of *IL6*, *IL1B*, *IL10*, *PDL1*, *IDO*, *SOCS1*, *SOCS3*, *RALDH1* and *RALDH2* by qPCR. Cell-free supernatants were used to quantify

TNFα, IL-6, IL-1β and IL-10 by ELISA. For inhibition experiments, hmoDCs were preincubated for 30 minutes with 1-MT (1 mM), rapamycin (100 nM), EDTA (0.5 mM), piceatannol (25 μM), U0126 (1 μM), SB202190 (1 μM), SP600125 (10 μM), or BAY117082 (2.5 μM); and for 1 hour with 2-DG (10 mM), heparin (100 IU/mL) or anti-IL-10 (2.5 μg/ml) (or corresponding vehicle controls or isotype control) prior to activation. Then, the cells were stimulated with Ca10 for 18 hours in the presence of the corresponding inhibitors to quantify TNFα, IL-6, IL-1β and IL-10 by ELISA or PD-L1 expression by flow cytometry. To analyse the contribution of the carbohydrate structure, hmoDCs were stimulated with 20 μg/mL of Ca10 (without treatment), Ca10 OX or Ca10 treated with Heparinase (HPSE treat.) for 18 hours. For blocking experiments with the monoclonal antibody A10, Ca10 was previously incubated during 1 hour with antibody A10, or corresponding isotype control, in a ratio of 1:10 or 1:5 (Ca10:A10) with continuous stirring, and then, it was added to hmoDCs for 18 hours.

Immature conventional hmoDCs or DCs differentiated in the presence of Ca10 (Ca10/hmoDCs) from healthy donors (10^6 cells/mL) were treated with medium (negative control) or LPS O127:B8 (100 ng/mL) for 18 hours at 37ºC in a humidified 5% CO_2 incubator. Cell pellets were used to assess their phenotype by flow cytometry using the following markers: HLA-DR, CD83, CD14, PD-L1 and ICOSL; and for the analysis of the mRNA levels of *PDL1*, *IDO*, *SOCS1*, *SOCS3*, *RALDH1* and *RALDH2* by qPCR. Cell-free supernatants were used to quantify TNFα, IL-6, IL-1β and IL-10 by ELISA.

In all cases, cell viability was analysed using Trypan blue in a Neubauer chamber under a light microscope.

5.2. Coculture experiments with naïve $CD4^+$ T cells

HmoDCs were cocultured with purified allogeneic naïve $CD4^+$ T cells (DC:T cell ratio of 1:5) for 3 days in the presence of control excipients, V132 (3 FTU per mL), MV140 (10^7 bact. per mL) or MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL) (**Figure 17A**). IFNγ, IL-5, IL-17A and IL-10 were quantified in cell-free supernatants by ELISA. Cell pellets were used to analyse T cell phenotype by flow cytometry using the following markers: CD3, CD4, CD127, CD25 and FOXP3. For proliferation assays, purified allogeneic naïve $CD4^+$ T cells were labeled with carboxy fluorescein succinimidyl ester (CFSE) (Molecular Probes) prior to coculture with immature hmoDCs. For intracellular staining, the primed $CD4^+$ T cells were washed and re-stimulated with 25 ng/mL PMA plus 1 mg/mL ionomycin (Sigma-Aldrich) for 6 hours, and 10 mg/mL of Brefeldin A (BD Bioscience) for the last 4 hours. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Cells

were stained with the combination of fluorochrome-labeled monoclonal antibodies (mAbs) to IFNγ, IL-10 and IL-17A.

HmoDCs or human total blood DCs stimulated with medium or Ca10 for 18 hours, as well as hmoDCs or Ca10/hmoDCs treated with medium for 18 hours, were cocultured with purified allogeneic naïve $CD4^+$ T cells (DC:T cell ratio of 1:5) for 5 days (**Figure 17B**). Inhibition assays were performed by adding anti-human IL-10 (2.5 µg/mL), anti-human PD-L1 (10 µg/mL) or corresponding isotype control to coculture. IFNγ, IL-5 and IL-10 were quantified in cell-free supernatants by ELISA. Cell pellets were used to analyse T cell phenotype by flow cytometry using the following markers: CD4, CD127, CD25 and FOXP3.

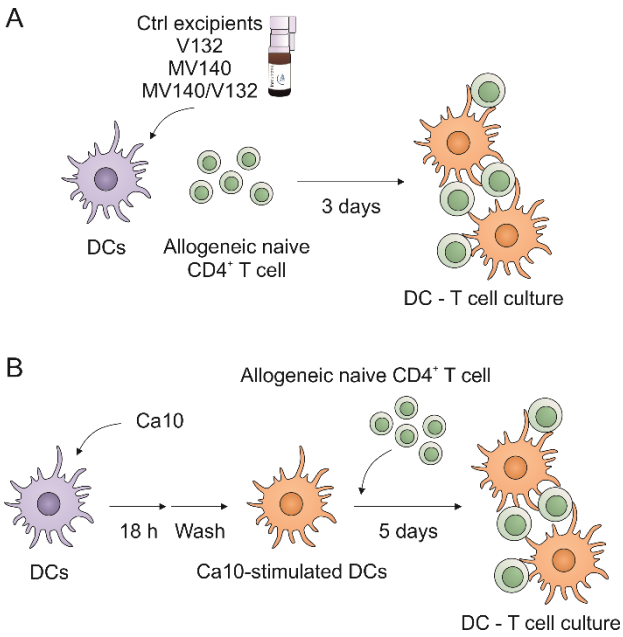

Figure 17. Human *in vitro* model for allogeneic coculture. (A) Coculture experiment of DCs and allogeneic naïve T cells in the presence of control (ctrl) excipients, V132, MV140 or MV140/V132. (B) DCs were stimulated with Ca10 and then, Ca10-activated DCs were coculture with allogeneic naïve T cells.

5.3. Regulatory T cell suppression assays

$CD4^+CD127^-CD25^{high}FOXP3^+$ regulatory T (Treg) cells induced by allogeneic Ca10-stimulated hmoDCs were purified by cell sorting of $CD4^+CD127^-CD25^{high}$ population. Purified Treg cells were mixed with CFSE-labelled autologous PBMC (responder cells) at different ratios and stimulated with plate-bound anti-human CD3 antibody (1 µg/mL, clone OKT3; eBioscience) and soluble anti-human CD28 (1 µg/mL, clone CD28.6; eBioscience) for 5 days. For control purposes, CFSE labeled PBMCs were cultured alone with or without stimulation, and the fraction of $CD4^-$ $CD127^-CD25^-$ cells (non-Treg cells) were also mixed with CFSE-labelled PBMCs. Proliferation of $CD4^+$ T cells was determined by CFSE dilution by flow cytometry.

5.4. Trained immunity assays

Trained immunity experiments were performed as previously described (251). 100 µL of human monocytes at 10^6 cells/mL or PBMC at 5 x 10^6 cells/mL density were added to a flat-bottom 96-well plates. Cells were incubated with control excipients, V132 (3 FTU per mL), MV140 (10^7 bact. per mL) or MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL) for 24 hours. Supernatants from monocytes were collected 24 hours after stimulation to quantify IL-6, TNFα, IL-1β, IL-10 and IL-1RA by ELISA. Cell viability was analysed by cytotoxicity assays. Cells were washed once with 200 µL of warm PBS and incubated for 6 days in RPMI culture medium Dutch modified with 10% human pooled serum. On day 6, supernatants were collected for lactate measurement. Cells were re-stimulated for 24 hours with LPS O155:B5 (10 ng/mL) or MV140 (10^7 bact. per mL), after which supernatants were collected to quantify IL-6, TNFα and IL-10 by ELISA (**Figure 18**).

Cell viability was analysed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), following manufacturer's recommendations. Briefly, released lactate dehydrogenase (LDH) upon cell lysis is measured with a 30-minute coupled enzymatic assay, which results in conversion of a tetrazolium salt (INT) into a red formazan product. The amount of colour formed is proportional to the number of lysed cells.

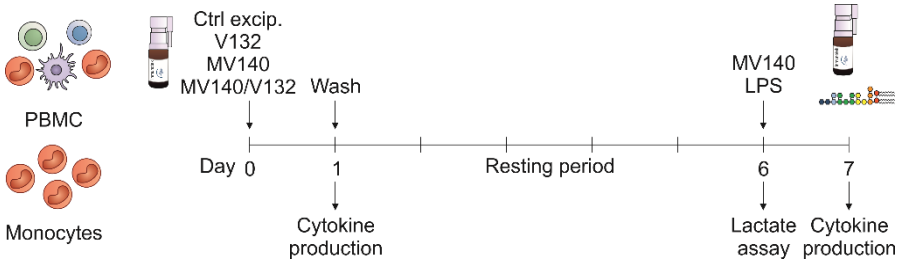

Figure 18. Human *in vitro* model for the induction of trained immunity in human PBMC and monocytes.

6. Flow cytometry

6.1. Surface staining

Cells were harvested, centrifuged at 300*g* during 5 minutes at room temperature and washed with PBS/2 mM EDTA/0.5% bovine serum albumin (BSA). Cells were suspended in 50 µL of PBS/2 mM EDTA/0.5% BSA and stained for 15 minutes at room temperature in the darkness with the corresponding fluorochrome-labelled antibodies were added (**Table 3**). Then, cells were washed with 1 mL of PBS/2 mM EDTA/0.5% BSA and centrifuged at 300*g* during 5 minutes at room temperature. The corresponding isotype controls were included in each staining. Cells were then fixed with 0.1%

PBS/PFA for flow cytometry analysis in the FACScalibur (Beckton Dickinson) at CCMF from UCM.

6.2. Intracellular staining

After T cell surface staining with anti-human CD4-PerCP and CD3-APC, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) for 20 minutes in darkness at ice. Then, cells were washed twice with Perm/Wash buffer (BD Biosciences) and incubated with fluorochrome-labelled mAbs specific for cytokines (IFNγ, IL-10 and IL-17A) for 30 minutes at room temperature in darkness (**Table 3**). The corresponding isotype controls were included in each staining. Cells were then fixed with 1% PBS/PFA for flow cytometry analysis in the FACScalibur (Beckton Dickinson) at CCMF from UCM.

Table 3. Flow cytometry antibodies.

Name	Supplier	Clone	Conjugate
Human FcR Blocking	Miltenyi	-	-
Human HLA-DR	Biolegend	L243	FITC, APC
Human CD86	Miltenyi	FM95	PE
Human CD83	Miltenyi	HB15	APC
Human CD14	BioLegend	RMO52	PerCP
Human CD274 (PD-L1)	BioLegend	MIH2	FITC
Human CD275 (ICOSL)	BD Pharmingen	2D3/B7-H2	PE
Human FOXP3	Biolegend	259D	Alexa488
Human CD127	Biolegend	A019D5	PE
Human CD4	Biolegend	OKT4	PerCP
Human CD25	Biolegend	BC96	APC
Human CD3	BD Pharmingen	OKT3	APC
Human IFN-γ	Biolegend	4S.B3	Alexa488
Human IL-17A	Biolegend	BL168	Alexa488
Human IL-10	Biolegend	JES3-19F1	PE
Human CD1c	Biolegend	L161	FITC
Human CD303	Miltenyi	AC144	PE
Human CD19	Biolegend	HIB19	PE/Cy7
Mouse FOXP3	Biolegend	MF-14	Alexa488
Mouse CD25	Biolegend	PC61	PE
Mouse CD4	Biolegend	GK1.5	PerCP

6.3. Intranuclear staining

After T cell surface staining with anti-human CD127-PE, CD4-PerCP and CD25-APC to analyses FOXP3 expression in human T cells primed with DCs, cells were fixed and permeabilized with FOXP3 Fix/Perm buffer (BioLegend) for 20 minutes in darkness at room temperature. A second permeabilization was carried out with FOXP3 Perm/Wash buffer (BioLegend) for 15 minutes in darkness at room temperature. Then, cells were stained with anti-human FOXP3-Alexa Fluor 488 for 30 minutes in darkness at room temperature. The same protocol was carried out for the characterization of $CD4^+CD25^{high}FOXP3^+$ Treg cells in freshly isolated mice splenocytes using anti-mouse CD4-PerCP and CD25-PE antibodies for surface staining and FOXP3-Alexa Fluor 488 antibody for intranuclear staining (**Table 3**). The viability dye eFluor 660 (eBioscience) was used for assess viability by flow cytometry and death cells were excluded in the analysis. The corresponding isotype controls were included in each staining. Cells were then fixed with 1% PBS/PFA for flow cytometry analysis in the FACScalibur (Beckton Dickinson) at CCMF from UCM.

6.4. CFSE staining

Human $CD4^+$ T cells were labelled with CFSE before coculture experiments. For that, CFSE probe (10 µmol/L, Molecular Probes) was added to the cells for 10 minutes in darkness at room temperature. Then, cold cRPMI media was added and cells were incubated for 5 minutes on ice. Cells were washed 3 times with media and were cocultured for 3 days with hmoDCs and the corresponding stimuli. The same protocol was carried out for the analysis of the proliferation of $CD4^+$ T cells in mice splenocytes using anti-mouse CD4-PerCP antibody for surface staining. The corresponding isotype control was included in each staining. Cells were then re-suspended in PBS/2 mM EDTA/0.5% BSA for flow cytometry analysis in the FACScalibur (Beckton Dickinson) at CCMF from UCM.

7. Cytokine quantification by ELISA

Concentrations of human IL-6, TNFα, IL-1β and IL-10 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences or RD Systems). Human IL-5, IL-10 and IFNγ, and mouse IL-6 and IFNγ were quantified by specific ELISA cytokine kits (BD Biosciences). Concentrations of human TGFβ1, IL-17A and IL-1RA, and mouse IL-17A were quantified by specific ELISA cytokine kits (R&D Systems). Human IL-23 and mouse TNFα and IL-10 concentration were quantified by specific ELISA cytokine kits (Invitrogen). In all cases, manufacturer's

instructions were followed with minor modifications. Briefly, the catching mAbs were coated onto microtiter plates that were blocked with assay diluent (PBS with 5% FBS) and incubated with the standards or samples. After washing, biotinylated anti-human or anti-mouse mAbs and streptavidin-labelled peroxidase were added to detect bound cytokines. After washing, chromogenic substrate (0.63 mg/mL o-phenylenediamine dihydrochloride (OPD), 0.03% H_2O_2 in 1 M sodium citrate) was added. The colour reaction was stopped by adding 3 N H_2SO_4 and the optical density (OD) values were measured at 492 or 450 nm according to each kit.

8. Western blot

Freshly isolated hmoDCs were treated with control excipients, V132 (3 FTU per mL), MV140 (10^7 bact. per mL), MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL), medium or Ca10 (20 µg/mL). After 30 minutes at 37°C, cells were harvested and lysed with RIPA buffer (ThermoFisher Scientific) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling) for 30 minutes at 4°C with vortexing every 10 minutes. Lysates were centrifuged at 10000*g* for 15 minutes and 4°C, and supernatants were collected for protein quantification with Micro BCA Kit (Pierce), following the manufacturer's instructions. 10 µg of total protein from cell lysates were separated by means of SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with primary antibodies (**Table 4**). Then, the membrane was washed and incubated with goat anti-rabbit (1:3000; Bio-Rad Laboratories) or goat anti-mouse (1:2500, Pierce) conjugated with horseradish peroxidase as a secondary antibody. Reactive bands were visualized with the ECL chemiluminescence system (Bio-Rad Laboratories). The OD of the reactive bands was quantified with Fujifilm Multi Gauge software, and values were expressed relative to the β-actin loading control.

Table 4. Western blot primary antibodies.

Name	Supplier	Clone	Dilution
Phospho-ERK (Thr202/Tyr204)	Cell Signaling	D13.14.4E	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling	G9	1:1000
Phospho-p38 MAPK (Thr180/182)	Cell Signaling	D3F9	1:1000
Phosho-IKKα/β (Ser176/177)	Cell Signaling	C84E11	1:1000
Phospho-IKBα (Ser32/35)	Cell Signaling	5A5	1:1000
Phospho-Akt (Ser473)	Cell Signaling	D9E	1:1000
Phospho-p70 S6 kinase (Thr389)	Cell Signaling	#9205	1:500
β-Actin	Sigma-Aldrich	AC-15	1:15000

9. RNA extraction and complementary DNA synthesis

RNA was isolated from harvested cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For mRNA analysis, cDNA was generated with a PrimeScript RT reagent Kit (Takara Bio).

10. Real-time quantitative PCR

Real-time quantitative PCR was performed on cDNA by using FastStart Universal SYBR Green Master (Roche). The sequences of the used pair primers are shown in **Table 5**. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). Data were normalized to elongation factor 1A (*EF1A*) and displayed as $2^{-\Delta CT}$ values multiplied by 10^4, with Δ cycle threshold (ΔCT) defined as the difference between the CT value for the gene of interest and *EF1A*. For titration experiments of Ca10 at different times, data were normalized to *EF1A* and displayed as fold change ($2^{-\Delta\Delta CT}$) relative to cells at 0 h (unstimulated).

Table 5. Primers for real-time qPCR.

Gene	Forward	Reverse
EF1A	CTGAACCATCCAGGCCAAAT	GCCGTGTGGCAATCCAAT
COXII	CTATCCTGCCCGCCATCATC	GGGATCGTTGACCTCGTCTG
GAPDH	CTGCACCACCAACTGCTTAGC	TCATGTTCTGGAGAGCCCCG
HIF1A	TCGCATCTTGATAAGGCCTCT	ACAAAACCATCCAAGGCTTTCA
IL6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL1B	TTTTTGCTGTGAGTCCCGGAG	TTCGACACATGGGATAACGAGG
IL10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT
PDL1	AAGATGAGGATATTTGCTGTCTTTATATTC	GTCCTTGGGAACCGTGACAGT
IDO	AGAAGTGGGCTTTGCTCTGC	TGGCAAGACCTTACGGACATCTC
SOCS1	CCCTGGTTGTTGTAGCAGCTT	CAACCCCTGGTTTGTGCAA
SOCS3	CCTCAGCATCTCTGTCGGAAGA	GCATCGTACTGGTCCAGGAACT
RALDH1	CTGCCGGGAAAAGCAATCT	AAATTCAACAGCATTGTCCAAGTC
RALDH2	AGGGCAGTTCTTGCAACCAT	GCGTAATATCGAAAGGTTTTGATGA
IL6-1 ChIP	TCGTGCATGACTTCAGCTTT	GCGCTAAGAAGCAGAACCAC
IL6-2 ChIP	AGGGAGAGCCAGAACACAGA	GAGTTTCCTCTGACTCCATCG
TNFA1 ChIP	AGAGGACCAGCTAAGAGGGA	AGCTTGTCAGGGGATGTGG
TNFA2 ChIP	GTGCTTGTTCCTCAGCCTCT	ATCACTCCAAAGTGCAGCAG
GAPDH ChIP	CCCCGGTTTCTATAAATTGAGC	AAGAAGATGCGGCTGACTGT
MYT1 ChIP	TTGGGGTTTGGAATTCTCTG	CAGTCTTTGTTCTCCGCACA
ZNF UTR ChIP	AAGCACTTTGACAACCGTGA	GGAGGAATTTTGTGGAGCAA

11. Metabolic studies

11.1. Metabolic studies in DCs

The Warburg effect in stimulated hmoDCs cultures was determined photometrically 18 hours after stimulation by quantifying the OD at 570 nm and calculating the Warburg effect as 1/OD570nm normalized to the corresponding condition. Glucose concentrations in cell-free culture supernatants were determined 18 hours after stimulation by using the Glucose (GO) Assay Kit (Sigma-Aldrich). The metabolic rate was derived mathematically in percentage of medium without cells (glucose concentration in RPMI 1640 = 2 mg/mL). Lactate concentrations in cell-free culture supernatants were determined 18 hours after stimulation by using the colorimetric L-Lactate Assay kit (Sigma-Aldrich), according to the manufacturer's recommendations. To measure mitochondrial membrane potential, MitoTracker Red CMXRos was used (250 nM, Thermo Fisher Scientific). Arbitrary fluorescence values were obtained in a FLUOstar Omega microplate reader (Ex/Em = 579/599 nm).

For real-time metabolic characterization, the extracellular acidification rate (ECAR, mpH/min), mitochondrial oxygen consumption rate (OCR, pmol/min), glycolytic proton efflux rate (glycoPER, pmol/min) were analysed using a Seahorse XF HS Mini Analyzer (Agilent). HmoDCs after Ca10 stimulation and freshly Ca10/hmoDCs were harvested, washed and resuspended in DMEM medium (Agilent) supplemented with 10 mM glucose (Sigma-Aldrich), 1 mM pyruvate (Life Technologies) and 2 mM glutamine (Sigma-Aldrich). Cells (25 x 10^3/well) were plated in poly-D-lysine-coated 8-well miniplates and incubated in a non-CO_2 incubator for 1 hour at 37ºC. ECAR and OCR were analysed using a Glycolysis Rate Assay with final concentrations of 1 µM rotenone and 1 µM antimycin A, and 50 mM 2-DG. A complete glycoPER study was performed in two consecutives stages: basal glycolysis (without drugs) and electron transportation chain inhibition (rotenone and antimycin A). OCR was analysed using a Cell Mito Stress Test with final concentrations of 1.5 µM oligomycin, 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 µM rotenone and 0.5 µM antimycin A. A complete OCR study was performed in four consecutive stages: basal respiration (without drugs), mitochondrial complex V inhibition (oligomycin), maximal respiration induction (FCCP), and electron transportation chain inhibition (rotenone and antimycin A).

11.2. Metabolic studies in trained PBMC and monocytes

Lactate concentrations in cell-free supernatants from PBMC or monocyte cultures were measured 24 hours after treatment with control excipients, V132, MV140 or MV140/V132, using a Lactate Fluorometric Assay Kit. Briefly, lactate was oxidized when lactate oxidase was added (Sigma-Aldrich) resulting in H_2O_2 which was coupled to the conversion of Amplex Red (Invitrogen) reagent to fluorescent resorufin by horseradish peroxidase (HRP) (ThermoFisher Scientific). Fluorescence values were obtained in a Synergy™ HT Multimode Microplate reader (BioTek) (filter 530/±25 and 590/±35).

For real-time metabolic experiments, adherent monocytes were obtained by incubating 5 x 10^7 PBMC in 10 cm Petri dishes for 1 hour. The adherent monocytes were treated with 10 mL of RPMI culture medium Dutch modified containing control excipients, V132 (3 FTU per mL), MV140 (10^7 bact. per mL) or MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL) for 24 hours, washed once with warm PBS and incubated in RPMI culture medium Dutch modified with 10% human pooled serum at 37ºC, 5% CO_2. Following 6 days in culture, cells were detached with Versene solution (ThermoFisher Scientific) and 5 x 10^5 cells were plated to overnight-calibrated cartridges in DMEM medium (Agilent) supplemented with 1 mM glutamine (Sigma-Aldrich) (pH adjusted to 7.4) and incubated for 1 hour in a non-CO_2-corrected incubator at 37ºC. ECAR and OCR in response to 10 mM glucose, 1 µM oligomycin, 1 µM FCCP, and 1.25 µM rotenone and 1.5 µM antimycin A injections were measured using a Seahorse XFe96 Analyzer (Agilent). A complete ECAR study was performed in two consecutive stages: basal extracellular acidification (after glucose injection), and mitochondrial complex V inhibition (oligomycin). A complete OCR study was performed in four consecutive stages: basal respiration (without drugs), mitochondrial complex V inhibition (oligomycin), maximal respiration induction (FCCP), and electron transportation chain inhibition (rotenone and antimycin A).

12. Chromatin immunoprecipitation (ChIP) assays

For real-time metabolic experiments, adherent monocytes were obtained by incubating 5 x 10^7 PBMC in 10 cm Petri dishes for 1 hour. The adherent monocytes were treated with 10 mL of RPMI culture medium Dutch modified containing control excipients, V132 (3 FTU per mL), MV140 (10^7 bact. per mL) or MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL) for 24 hours, washed once with warm PBS and incubated in RPMI culture medium Dutch modified with 10% human pooled serum at 37ºC, 5% CO_2. Following 6 days in culture, cells were detached with Versene solution (ThermoFisher Scientific) and fixed in 1% methanol-free formaldehyde. Afterward, cells were sonicated

using a Diagenode Bioruptor UCD-300 for 10 minutes (30 seconds on; 30 seconds off). 33 µL of the solution containing chromatin was incubated with 255 µL of dilution buffer, 12 µL of protease inhibitor cocktail (Roche) and 1 µg of H3K27ac (C15410196) or H3K9me3 (C15410193) antibodies (Diagenode), overnight at 4ºC with rotation. Protein A/G magnetic beads (10001D and 10003D, Invitrogen) were washed in dilution buffer with 0.15% SDS and 0.1% BSA, added to the chromatin/antibody mix and rotated for 60 minutes at 4ºC. Beads were washed with 500 µL buffer for 5 minutes at 4ºC with five rounds of washes. After washing chromatin was eluted using elution buffer for 20 minutes. Supernatant was collected, 8 µL 5 M NaCl and 1 µL proteinase K (Qiagen) were added, and samples were de-crosslinked at 64ºC for 4 hours. DNA was isolated with the MinElute PCR Purification Kit (Qiagen), following manufacturer's protocol. Real-time quantitative PCR was performed on the purified ChIP and INPUT DNAs using the SYBR Green method (Roche). Samples were analysed by a comparative Ct method according to the manufacturer's instructions. Sequences of the used pair primers are in **Table 5**. *GAPDH* gene was used as positive and negative control for H3K27ac and H3K9me3, respectively; *MYT1* gene was used as negative control for H3K27ac; and *ZNF UTR* was used as positive control for H3K9me3.

13. Animal models

13.1. Sublingual immunization mouse model with MV140/V132, MV140, V132 or control excipients

BALB/c mice (6 weeks old, Charles River) were immunized four times with 20 µL of control excipients, MV140/V132, MV140 alone or V132 alone (300 FTU per mL) by sublingual administration every 7 days and sacrificed 7 days after the last immunization. Spleens and plasma samples were collected to assess the proliferation of splenic CFSE-labeled $CD4^+$ T cells and serum specific immunoglobulin (Ig) levels, respectively.

13.2. V132 immunization for trained immunity mouse model

BALB/c mice (female, 6 weeks old, Charles River) were intraperitoneally immunized with 20 µL of control excipients or V132 (300 FTU per mL) at day 0 and 3 and sacrificed 4 days after the last immunization. In another animal model, BALB/c mice (female, 6 weeks old, Charles River) were sublingually immunized for 5 consecutive days during 2 weeks with 20 µL of control excipients or V132 (300 FTU per mL) and sacrificed 3 days after last immunization. Moreover, some immunized mice were sublingually administered with 20 µL of MV140 (10^9 bact. per mL) 3 days after last sensitization and sacrificed after 8

days of the MV140 challenge. Peritoneal cells and spleens were collected for *in vitro* stimulation to assess cytokine production.

13.3. *In vivo* Ehrlich tumor growth

ET cells, initially originated from hyperdiploid Ehrlich-Lettré mouse ascites tumor cells, are derived from a cloned cell variant selected for its high reactivity with the monoclonal antibody A10. C57BL/6J mice (6 weeks old, Charles River) were inoculated with 10^5 ET cells by subcutaneous injection into the left groin. Tumor size was monitored weekly by calculating the tumor area (mm^2) as the product of long (mm) and wide (mm) measured with Vernier calipers. Mice were sacrificed at different days (22, 32, 42, and 58 days) of tumor development. After sacrifice, the average of all tumor size was calculated (117 mm^2) allowing the stratification of mice bearing small (below the average, <117 mm^2) and large (above the average, >117 mm^2) tumors. In another animal model, C57BL/6J mice were alternatively intravenously and intraperitoneally administered with Ca10 (40 µg/mL) in the presence or absence of antibody A10 (80 µg/mL) for 7 days, and sacrificed the next day after the last immunization. Spleens and blood, collected by cardiac puncture, were obtained to assess Treg cells and circulating Ca10, respectively.

13.4. Plasma collection and ELISA of induced serum-specific Ig

Mice were bleeding and blood was collected using microtainer tubes. Samples were centrifuged (10000*g*, 10 minutes) and plasma was collected and stored at -80 ºC for further analysis. Serum specific IgG, IgA, IgG1, and IgG2a for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* induced after sublingually immunization were determined by ELISA. Briefly, 96-well nontissue culture-treated plates were pretreated with poly-L-lysine (Sigma-Aldrich) for 1 hour under UV light and coated with each of the heat-inactivated whole cell bacteria or yeast (300 FTU per mL) overnight at 4°C, and subsequently, incubated with mouse serum dilutions for 2 hours at room temperature. Specific immunoglobulins were detected with biotin rat anti-mouse IgA and IgG (both from Sigma-Aldrich). Signal was developed by incubation with HRP (Sigma-Aldrich). Peroxidase activity was revealed by the addition of OPD (Sigma-Aldrich). The colour reaction was stopped with 1 N HCl and the OD values were measured at 492 nm.

13.5. Spleen processing

Spleens were minced and filtered through 40 µm nylon cell strainers to obtain a single-cell suspension. Then, red blood cells were lysed with ACK lysis buffer before being resuspended in cRPMI medium. Splenocyte viability was assessed using Trypan blue in a Neubauer chamber under a light microscope.

13.5.1. Splenocyte culture for proliferation assays

Splenocytes from mice sublingually immunized with control excipients or MV140/V132 were labelled with CFSE (5 mM, Life technologies) and stimulated *in vitro* with V132 (6 FTU per mL), MV140 (6 FTU per mL), MV140/V132 (MV140, 3 FTU per mL, and V132, 3 FTU per mL), phytohaemagglutinin (PHA, 5 µg/mL, Sigma-Aldrich) or control excipients for 5 days. Proliferation of CFSE-labeled $CD4^+$ T cells was monitored by flow cytometry.

13.5.2. Splenocyte culture for cytokine production

Spleens from mice immunized with V132 for trained immunity assays were processed and triplicates of 0.8 x 10^6 splenocytes were cultured *in vitro* with control excipients or MV140 (10^5-10^7 bact. per mL) in flat-bottom 96-well plates. After 24 or 72 hours of culture at 37ºC and 5% CO_2, the triplicates were pooled and cell-free supernatants were used to quantify IL-6, TNFα, IL-17, IFNγ and IL-10 by ELISA.

13.5.3. Splenocytes for Treg cell assays

Freshly isolated splenocytes were used for analysis of the presence of splenic $CD4^+CD25^{high}FOXP3^+$ Treg cells in the different ET-bearing mice, mice administered with Ca10 and control mice was quantified, as described above.

13.6. Peritoneal lavage and peritoneal cell culture

Peritoneal cells were collected with PBS/2mM EDTA and resuspended in cRPMI medium. Duplicates of 0.25 x 10^6 peritoneal cells were cultured *in vitro* with control excipients or MV140 (10^6 bact. per mL) in flat-bottom 96-well plates for 24 hours at 37ºC and 5% CO_2. Cell-free supernatants of duplicates were pooled and used to quantify IL-6 and TNFα.

13.7. Ca10 measurement by ELISA

A sandwich ELISA was developed using A10, the Ca10-defining monoclonal antibody obtained against ET cell surface carbohydrates, to analyses Ca10 levels in mouse sera. Purified A10 (IgM*k*), from culture supernatant of A10-producing hybridoma, was used as capture and detection antibody once labeled with horseradish peroxidase (HRP-A10). Plates were coated overnight at 4°C with 5 µg/mL of A10 in carbonate buffer. Then, they were washed with PBS/0.25% Tween-20 and incubated 2 hours at room temperature with the samples diluted in PBS/0.25% Tween-20. After a wash step, a dilution of HRP-A10 in PBS/0.25% Tween-20/5% FBS was added and incubated for 2 hours at room

temperature. After a final washing, the peroxidase substrate (OPD) solution was added, the enzymatic reaction allowed to develop for 30 minutes and stopped with HCl. Ca10 concentration was expressed as arbitrary units (AU) per mL by extrapolating the OD at 495 nm to a reference curve established with Ca10.

14. Statistical analysis

In all experiments, data represent the mean ± SEM of the indicated parameters. Statistical analysis was calculated using paired or unpaired *t* test, Wilcoxon matched-pairs test, Mann-Whitney test, One-way ANOVA or Spearman correlation test. Statistical analyses were performed using GraphPad Prism software, version 6.0. Significance was defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

RESULTS AND DISCUSSION

Block A: Study of the molecular mechanisms involved in the mode of action of fungal vaccine V132 and polybacterial preparation MV140 for recurrent genitourinary tract infections

Objective 1. Study of the molecular mechanisms involved in the capacity of MV140/V132 to immunomodulate human DCs

The widespread use of antibiotics for recurrent urinary tract infections (RUTIs) is linked with antibiotic resistances and vaginal microbiota deleterious effects (135, 137, 145). Alterations in vaginal microbiota facilitate *C. albicans* overgrowth thus increasing the risk to vulvovaginal candidiasis (VVCs) and the overused of antifungals to avoid recurrences (133, 136, 138). Sublingual vaccines have emerged as useful approaches to improve non-antibiotics and non-antifungal therapeutic strategies. The sublingual polybacterial preparation MV140 is composed of whole heat-inactivated bacteria causing the majority of RUTIs in Europe: 75% Gram-negative bacteria (25% *E. coli*, 25% *P. vulgaris* and 25% *K. pneumoniae*) and 25% Gram-positive bacteria (*E. faecalis*). MV140 significantly reduced the infection rates in patients with RUTIs (152, 192-198), acting directly on human DCs promoting Th1/Th17 and IL-10 immune responses (78). V132 is a sublingual fungal preparation based on whole heat-inactivated *C. albicans*. It has been developed as an alternative strategy for recurrent VVCs (RVVCs), but the detailed molecular mechanisms involved in the mode of action still remains unknown. Given the tight relationship between RUTIs and RVVCs, a polymicrobial formulation, via combination of MV140 and V132, might well represent a suitable vaccine for both diseases. In this chapter, we investigate the mode of action of V132, MV140 and the combination of both of them to immunomodulate human DCs.

1.1. To study the capacity of MV140/V132 to regulate the phenotype and function of human DCs

To assess the capacity of the combination of the polyvalent bacteria preparation MV140 and the fungal vaccine V132 to modulate the phenotype and function of human DCs, human monocyte-derived DCs (hmoDCs) were generated as previously described in Materials and Methods and stimulated with V132 alone, MV140 alone, the combination of both of them (MV140/V132) or control excipients (containing all excipients except the microbial components) for 18 hours.

Firstly, the expression levels of the activation surface markers HLA-DR, CD83 and CD86 were analysed by flow cytometry. After antigen recognition, DCs mature and upregulate the expression of MHC class II and costimulatory molecules to induce T cell responses (31). DCs present the antigens to $CD4^+$ T cells as peptide-MHC class II (also called ‘HLA-DR’ in humans) complex (28). Furthermore, activation of $CD4^+$ T cells requires the binding of costimulatory molecules such as CD86 and CD83 expressed by DCs after antigen recognition (83, 252).

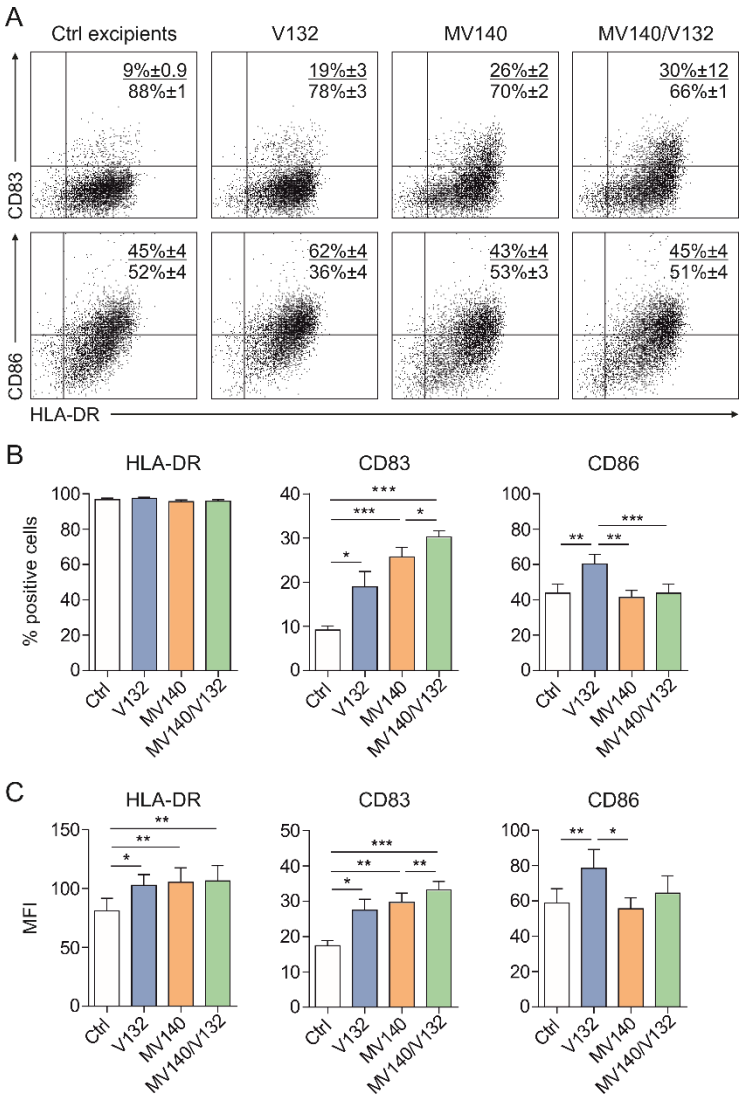

Figure 19. MV140/V132 induces maturation of hmoDCs. (A) Flow cytometry representative dot plots for the expression of surface markers HLA-DR, CD83 and CD86 after stimulation of hmoDCs with control excipients (ctrl), V132, MV140 or MV140/V132 for 18 hours. (B) Percentage of HLA-DR-, CD83- and CD86-positive cells after stimulation (n = 7-8). (C) Mean fluorescence intensity (MFI) values for HLA-DR, CD83 and CD86 (n = 7-8). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

As shown in **Figure 19A and B**, significant changes were not detected in the percentage of cells expressing HLA-DR and as expected for DCs, almost in all assayed conditions were HLA-DR$^+$. However, the expression levels (MFI values) were significantly increased after V132, MV140 or MV140/V132 stimulation compared to control excipients, without significant differences among them (**Figure 19C**). V132, MV140 and MV140/V132 significantly increased the expression of the co-stimulatory molecule CD83 in hmoDCs compared to control excipients (**Figure 19A, B and C**). MV140 and MV140/V132 induced a higher expression of CD83 than V132 without

significant differences (**Figure 19B and C**). Both the percentage of positive cells and the expression levels of CD83 were significantly higher in hmoDCs stimulated with MV140/V132 than MV140, indicating that the combination of these stimuli in a single formulation significantly enhance the maturation of hmoDCs (**Figure 19B and C**). The expression of CD86 was significantly higher in hmoDCs stimulated with V132 than the other stimuli, but no differences were observed after MV140 and MV140/V132 stimulation compared to control excipients (**Figure 19A, B and C**). These data demonstrate that V132, MV140 and MV140/V132 promote the maturation and activation of hmoDCs.

Then, the cytokine signature after stimulation with V132, MV140, MV140/V132 or control excipients was quantified by ELISA. HmoDCs treated with V132 produced significantly higher levels of IL-6 and TNFα and a slight increase in the production of IL-1β and IL-10 than control excipients. IL-23 levels were no detectable in cell-free supernatants from V132-treated hmoDCs (**Figure 20**). MV140 induced a significant production of IL-6, TNFα, IL-1β, IL-23 and IL-10 compared to control treatment, as previously described (78), and also compared to V132 (**Figure 20**). MV140/V132-activated hmoDCs induced a significantly higher production of pro-inflammatory cytokines and IL-10 than control excipients- or V132-activated hmoDCs (**Figure 20**). Interestingly, MV140/V132 stimulation induced a significantly higher concentration of TNFα, IL-23 and IL-10 than MV140 in hmoDCs (**Figure 20**).

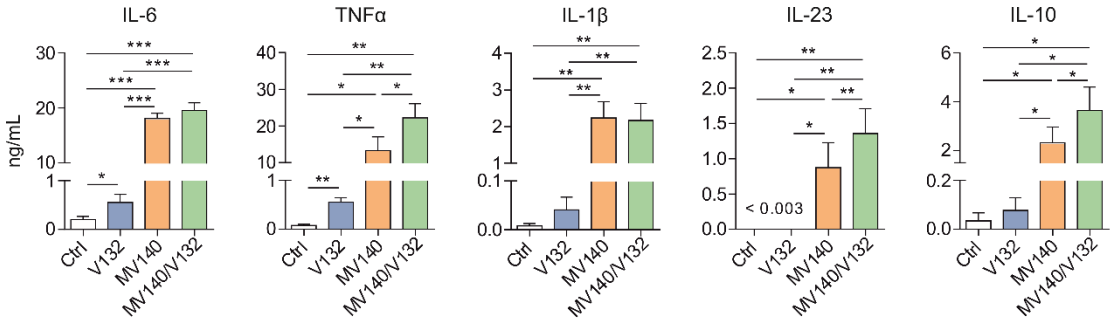

Figure 20. MV140/V132 induces the production of pro-inflammatory cytokines and IL-10 in hmoDCs. Cytokine production after stimulation of hmoDCs with control excipients (ctrl), V132, MV140 or MV140/V132 for 18 hours quantified in cell-free supernatants by ELISA (n = 6-8). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

To test whether simultaneous stimulation of hmoDCs with MV140/V132 enhances the production of TNFα, IL-23 and IL-10 in a synergistic manner, cytokine concentration induced by MV140/V132 showed in **Figure 20** were directly compared to those resulting from the addition of the cytokine levels produced by activation with MV140 and V132 separately (**Figure 20**). MV140/V132-activated hmoDCs induced

significantly higher levels of TNFα, IL-23 and IL-10 than the corresponding cytokine levels produced by V132- plus MV140-treated hmoDCs, supporting synergistic cooperation in the simultaneous stimulation with the combination of MV140 and V132 (**Figure 21**). Activation of DCs induces the secretion of a broad spectrum of pleiotropic cytokines with pro- and anti-inflammatory functions that are involved in the polarization of different T cell responses, among other functions (30, 87, 91). TNFα promotes Th1 responses and IL-6, IL-1β and IL-23 drive the polarization of Th17 cells, whereas the anti-inflammatory cytokine IL-10 is essential to avoid excessive immune responses and contributes to the generation of regulatory T (Treg) cells (87, 91). Our data revealed that MV140/V132 promotes a potent pro-inflammatory cytokine production (TNFα and IL-23) and higher levels of IL-10 in human DCs compared with individual MV140 or V132 stimulation (78), suggesting a Th1, Th17 and Treg cell polarization.

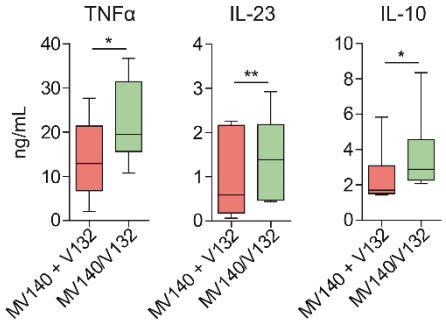

Figure 21. MV140/V132 induces a higher cytokine production than the individual components. Cytokine levels of V132-treated hmoDCs plus cytokine levels of MV140-treated hmoDCs compared with cytokine levels of MV140/V132-stimulated hmoDCs (n = 6). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Then, we assessed whether hmoDCs produced TGF-β1 after stimulation. TGF-β1 is a pleiotropic cytokine that is required for the polarization both Th17 and Treg cell subsets in cooperation with other cytokines (87, 91). However, we did not detect significant differences in the levels of TGF-β1 among any of the assayed conditions (**Figure 22**), suggesting that this cytokine is not involved in the polarization of Th17 and Treg subsets.

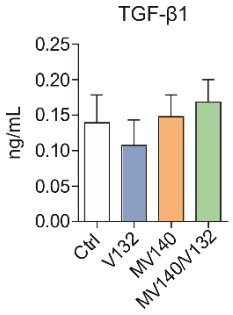

Figure 22. No significant differences in the concentration of TGF-β1 after stimulation of hmoDCs. TGF-β1 production after stimulation of hmoDCs with control excipients (ctrl), V132, MV140 or MV140/V132 for 18 hours quantified in cell-free supernatants by ELISA (n = 5). Values are mean ± SEM.

Collectively, our data showed that MV140/V132 promotes the maturation of human DCs with a higher expression of the costimulatory molecule CD83 as well as pro-inflammatory cytokine and IL-10 production than the individual components.

1.2. To study the polarization of T cell responses induced by human DCs stimulated with MV140/V132

After antigen recognition, DCs migrate to lymph nodes and present the processed antigens to naïve T cells to induce T cell differentiation (27, 28). Therefore, to determine the capacity of human DCs treated with the combination of MV140 and V132 to polarize $CD4^+$ T cell responses, we performed allogeneic coculture experiments. For that, we coculture allogeneic naïve $CD4^+$ T cells with hmoDCs in the presence of control excipients, V132, MV140 or MV140/V132. After 3 days of coculture, T cell proliferation was analysed by flow cytometry and T cell cytokine production was quantified by ELISA.

HmoDCs stimulated with MV140 and MV140/V132 induced a significantly higher percentage of proliferating allogeneic $CD4^+$ T cells than control hmoDCs (**Figure 23**). V132-activated hmoDCs were also able to stimulate proliferation of $CD4^+$ T cells, but significant changes were not detected when comparing with excipients-treated hmoDCs (**Figure 23**).

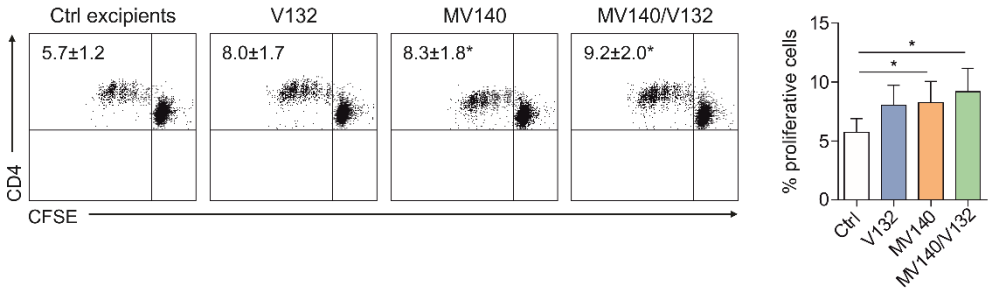

Figure 23. MV140/V132-activated hmoDCs promote T cell proliferation. Representative dot plots of proliferating CFSE-labeled allogeneic naïve $CD4^+$ T cells primed by hmoDCs in the presence of control excipients (ctrl), V132, MV140 or MV140/V132 after 3 days of coculture (left side) and the graph with the frequency of proliferating cells (right side) (n = 5). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. $*P < 0.05$.

As shown in **Figure 24**, hmoDCs activated with MV140 or MV140/V132 generated T cells producing significantly higher levels of IFNγ, IL-17A and IL-10 than control excipients- or V132-stimulated hmoDCs. We did not detect IL-5 production in any of the assayed conditions. Interestingly, MV140/V132-activated hmoDCs generated T cells producing significantly higher levels of IL-17A and IL-10 than MV140-activated hmoDCs (**Figure 24**).

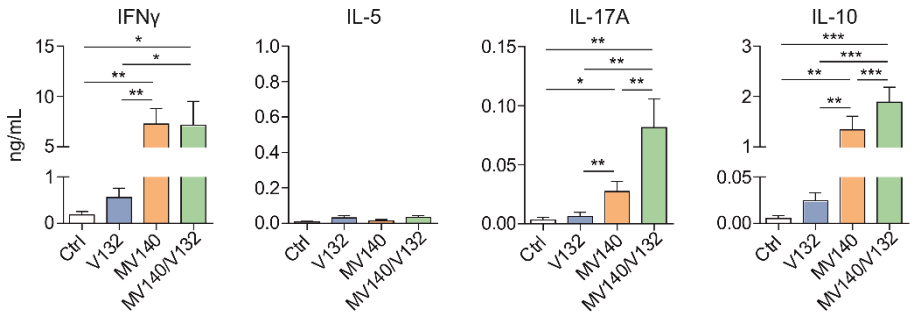

Figure 24. MV140/V132-activated hmoDCs induce T cells producing IFNγ, IL-17A and IL-10. Cytokines produced by allogeneic naïve CD4$^+$ T cells primed by hmoDCs in the presence of control excipients (ctrl), V132, MV140 or MV140/V132 after 3 days quantified in cell-free supernatants by ELISA (n = 8-12). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. *P < 0.05, **P < 0.01, and ***P < 0.001.

To assess potential synergistic effects, the levels of IL-17A and IL-10 produced by T cells generated by MV140/V132-activated hmoDCs (**Figure 24**) were compared to those resulting from the addition of these cytokine levels in MV140- and V132-stimulated conditions separately, shown in **Figure 24**. IL-17A and IL-10 production was significantly higher when T cells were primed by hmoDCs activated with MV140/V132 than the sum of the cytokine levels produced by T cells generated by V132- plus MV140-activated hmoDCs, thus supporting synergistic effects of MV140/V132 at the T cell level (**Figure 25**).

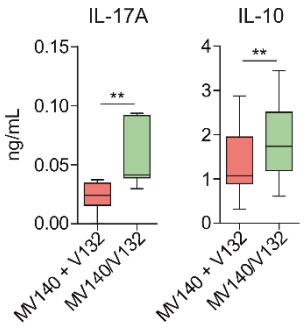

Figure 25. T cells primed by MV140/V132-activated hmoDCs induce a higher cytokine production than the individual components. Levels of IL-17A and IL-10 for V132- plus MV140-stimulated cocultures compared to MV140/V132-stimulated cocultures (n = 8-12). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P < 0.01.

To verify these data at the single cell level, we performed the same allogeneic coculture experiments and intracellular cytokine staining. The percentages of IFNγ-, IL-10- and IL-17A-producing CD4$^+$ T cells generated by hmoDCs treated with V132, MV140 or MV140/V132 were significantly higher than those produced by T cells generated by control excipients-treated hmoDCs (**Figure 26**). Supporting the previous results, MV140/V132-activated hmoDCs generated a significantly higher percentage of IL-10- and IL-17A-producing T cells than MV140-treated cells (**Figure 26**).

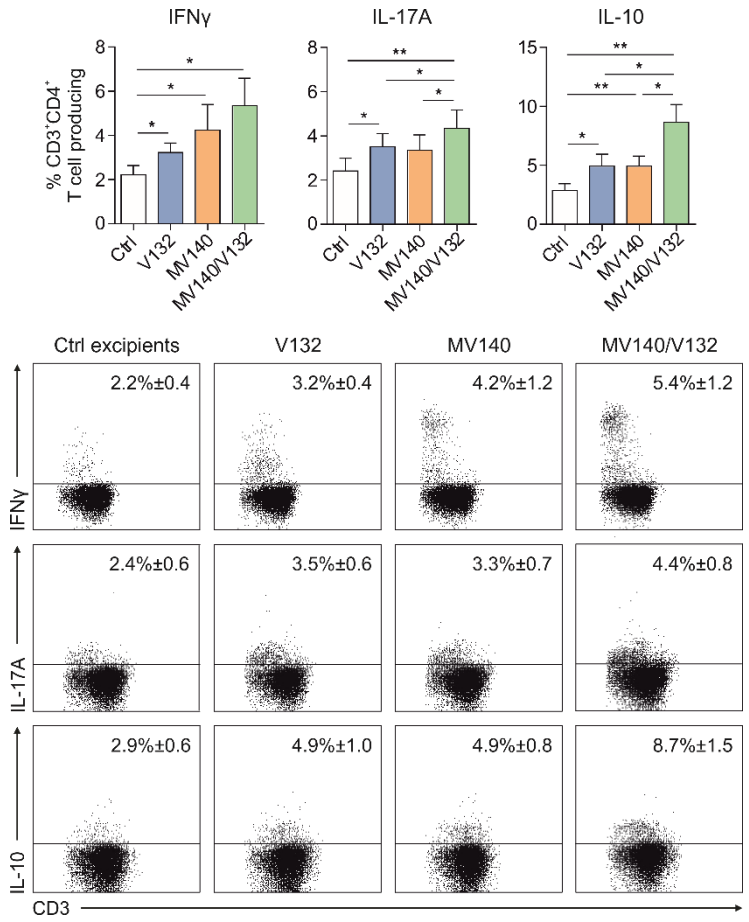

Figure 26. MV140/V132-activated hmoDCs generate IFNγ-, IL-17A- and IL-10-producing T cells. Percentage of $CD3^+CD4^+$ T cells producing IFNγ, IL-17A and IL-10 generated after 3 days of coculture with hmoDCs in the presence of control excipients (ctrl), V132, MV140 or MV140/V132 as determined by intracellular staining and flow cytometry analysis (n = 7-8). Representative dot plots of the intracellular staining are shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Then, to investigate the capacity of activated hmoDCs to induce Treg cells, we performed the same allogeneic coculture experiments and intranuclear FOXP3 staining. V132-, MV140- and MV140/V132-activated hmoDCs induced significantly higher percentages of $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells than excipients treated hmoDCs (**Figure 27**). Remarkably, the number of $FOXP3^+$ Treg cells generated by MV140/V132-activated hmoDCs was significantly higher than those induced by hmoDCs stimulated with V132 or MV140 alone (**Figure 27**).

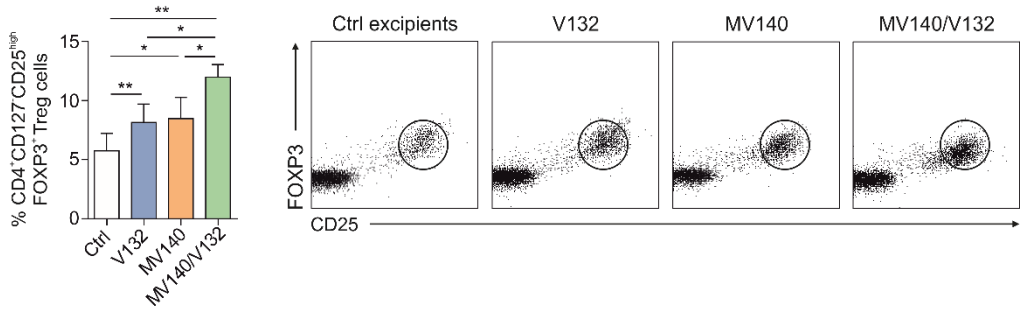

Figure 27. MV140/V132-activated hmoDCs generate FOXP3⁺ Treg cells. Percentage of induced $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells gated on $CD4^+$ T cells after 3 days of coculture with hmoDCs in the presence of control excipients (ctrl), V132, MV140 or MV140/V132 (n = 5). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Our data demonstrated that M140/V132 imprints DCs with the capacity to promote broad T cell responses. Firstly, potent IFNγ- and IL-17A-producing T cells mediate pathogen clearance and both T cell phenotypes favour effective immune responses from both bacteria causing UTIs and *C. albicans* in VVCs (132, 158). Th1 cells, essential for intracellular pathogens (bacteria or virus), are the main responsible for the IFNγ production that strongly activates the antimicrobial activity of macrophages, neutrophils and $CD8^+$ T cells (88, 90, 253). Th17 response is key against extracellular pathogens (bacteria and fungi) producing IL-17 for neutrophil recruitment, IL-21 for $CD8^+$ T cell, NK cell, B cell and DC activation, and IL-22 for the secretion of β-defensins (90, 253, 254). In addition, MV140/V132 also induces a higher IL-10 production in DCs and T cells than individual stimuli separately. IL-10-produging T cells and Treg cells are key for the control of the excessive activation of immune system avoiding chronic inflammation and host damage (37, 255, 256). Additionally, Treg cell generation enhances microbiota protection and tissue repair, which is particularly important to restore of healthy immune responses in urogenital infections after long-term antibiotic and antifungal treatments (257, 258).

1.3. To study the main immune signalling pathways involved in the immunomodulatory effects of MV140/V132 in human DCs

MV140 triggers Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) in human DCs, which culminate in the activation of mitogen-activated protein kinases (MAPKs)- and nuclear factor κB (NF-κB)-mediated signalling pathways (78). *C. albicans* also activates TLRs and CLRs (mainly dectin 1) leading the subsequent activation of MAPKs and NF-κB (259). To elucidate the molecular mechanisms underlying the immunomodulatory effects of MV140/V132, hmoDCs were stimulated with V132,

MV140, MV140/V132 or control excipients for 30 minutes and the activation of diverse immune signalling pathways was analysed by western blot. We assessed the phosphorylation of MAPK extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK) and p38. Additionally, we evaluated the activation of NF-κB signalling pathways as determined by the phosphorylation of IκB kinase complex (IKKα/β). V132 induced a rapid activation of the MAPK ERK1/2 (phosphorylation of Thr202/Tyr204) and the MAPK JNK (phosphorylation of Thr183/Tyr185) compared to control excipients without activation of MAPK p38 (phosphorylation of Thr108/Tyr182) or IKKα/β (phosphorylation of Ser176/177) (**Figure 28**). MV140 alone induced a significantly higher phosphorylation of p38 and IKKα/β than V132 alone in hmoDCs without significant changes observed for ERK or JNK compared to V132 alone (**Figure 28**). Interestingly, MV140/V132 induced a significantly higher phosphorylation of ERK, p38, and IKKα/β than V132 (**Figure 28**). However, a slight but non-significant increment in the activation of ERK and JNK was observed after MV140/V132 stimulation compared with MV140 alone without differences in the activation of p38 or IKKα/β (**Figure 28**).

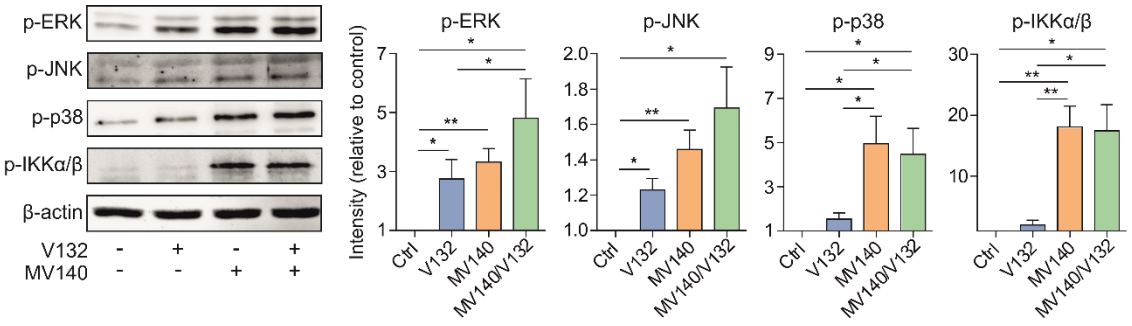

Figure 28. MV140/V132 activates MAPKs and NF-κB signalling pathways in hmoDCs. Western blot analysis of protein extracts from hmoDCs stimulated with control excipients (ctrl), V132, MV140 or MV140/V132 for 30 minutes (n = 4-7). Quantification of the reactive phosphorylated bands by scanning densitometry. β-actin was used as a loading control. One representative example is shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Sensing of *C. albicans* β-glucan by dectin-1 induces the activation of mammalian target of rapamycin (mTOR) signalling pathway (120), however, the implication of mTOR pathway in the mechanism of action of MV140 remains unknown. We evaluated mTOR signalling pathways as determined by the phosphorylation of mTOR upstream activator protein kinase B (Akt) and mTOR downstream substrate p70S6 kinase (p70S6K). As shown in **Figure 29**, V132, MV140 and MV140/V132 induced a significant activation of Akt (phosphorylation of Ser473) and p70S6K (phosphorylation of Thr389) compared to control excipients. V132 and MV140/V132 stimulation induced a similar and significantly higher phosphorylation of Akt and p70S6K than MV140 in hmoDCs (**Figure 29**).

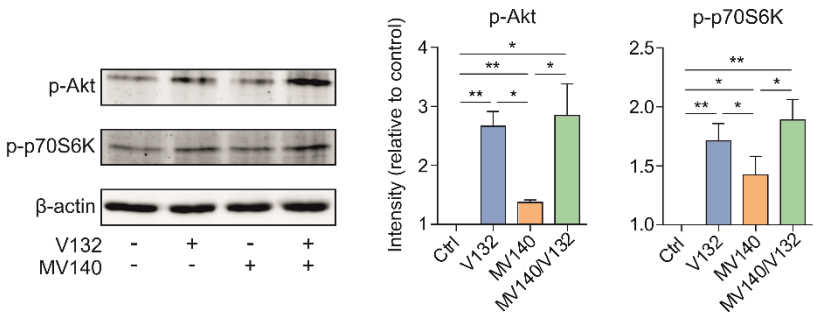

Figure 29. MV140/V132 activates mTOR signalling pathway in hmoDCs. Western blot analysis of protein extracts from hmoDCs stimulated with control excipients (ctrl), V132, MV140 or MV140/V132 for 30 minutes (n = 4-5). Quantification of the reactive phosphorylated bands by scanning densitometry. β-actin was used as a loading control. One representative example is shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Collectively, these data demonstrated that MV140/V132 induces MAPKs-, NF-κB- and mTOR-mediated signalling pathways that promote the orchestration of immune responses by inducing cytokine production (44, 52) and regulating metabolic reprogramming in DCs (107). Interestingly, only V132 alone and MV140/V132, but not MV140 stimulation, promote mTOR signalling pathway, thus indicating that V132 significantly contributes to the activation of this key pathway in trained immunity (120). According to our data, MV140 is the main driver of p38 and JNK within the MV140/V132 formulation, which are signalling that also contribute to acquire a trained phenotype in human monocytes (260).

1.4. To study metabolic and epigenetic changes induced by MV140/V132 in human DCs

mTOR plays a central role in the regulation of metabolic pathways in DCs, thus controlling cellular growth, proliferation, cytokine production and antigen presentation (107, 114). Therefore, we studied whether V132, MV140 or the combination of MV140 and V132 could rewire metabolism in hmoDCs. HmoDCs were stimulated with V132, MV140 and MV140/V132 for 18 hours and glucose consumption and lactate production were quantified in cell free supernatants. A slight non-significant increase in the consumption of glucose and metabolic rate was observed in hmoDCs after V132 stimulation compared to excipients-treated hmoDCs (**Figure 30A**). In contrast, the stimulation of hmoDCs with MV140 or MV140/V132 significantly enhanced the consumption of glucose from the culture medium and, therefore, the metabolic rate compared to excipients- or V132-treated hmoDCs, without significant differences among them (**Figure 30A**). Glucose is oxidized during glycolysis to obtain pyruvate, which can be metabolised to lactate for quick generation of energy, or acetyl-CoA to fuel the TCA

cycle and then, OXPHOS to obtain ATP (106). We firstly analysed the lactate production in cell-free supernatants. Interestingly, MV140/V132-activated hmoDCs significantly increased lactate production relative to MV140 stimulation (**Figure 30B**).

Then, to further explore the contribution of OXPHOS, the changes in mitochondrial membrane potential were determined using the MitoTracker Red CMXRos. The mitochondrial membrane potential was significantly lower in hmoDCs after MV140/V132 stimulation relative to treatment with MV140 alone (**Figure 30C**), supporting that the combination of MV140 and V132 displays low mitochondrial activity. To analyse the mitochondrial mass, we determined by qPCR the amount of mitochondrial DNA (*COXII*) and nuclear DNA (*GAPDH*). Then, the mitochondrial content was determined by calculating *COXII*/*GAPDH* ratio as previously described by Malinarich et al (115). As shown in **Figure 30D**, MV140/V132-stimulated hmoDCs displayed a significantly lower mitochondrial content than hmoDCs stimulated with MV140 alone, suggesting that DCs decreases both mitochondrial activity and content in the presence of MV140/V132. Collectively, these data suggested that the combination of MV140 and V132 shifts the metabolism from OXPHOS towards an increased glucose consumption and lactate production in a process similar to Warburg effect (109).

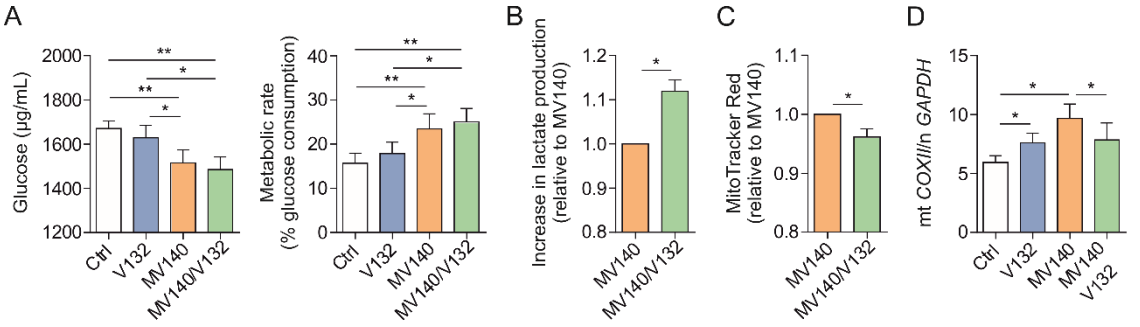

Figure 30. Analysis of metabolic changes induced by V132, MV140 or MV140/V132 in hmoDCs. (A) Glucose consumption by hmoDCs treated with control excipients (ctrl), V132, MV140 or MV140/V142 after 18 hours and calculated metabolic rate (percentage of glucose consumption) (n = 6). (B) Increase in lactate production in cell-free supernatants from MV140/V132-stimulated hmoDCs relative to MV140-activated hmoDCs (n = 4). (C) Fluorescence intensity of MV140/V132-stimulated hmoDCs relative to MV140-activated hmoDCs stained with MitoTracker Red (n = 7). (D) Mitochondrial mass determination as the ratio of mitochondrial (mt) and nuclear (n) DNA as expressed by mt *COXII*/n *GAPDH* (n = 4). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. *$P < 0.05$, and **$P < 0.01$.

HIF-1α is a downstream transcription factor activated by mTOR acting as a regulator of metabolism. HIF-1α promotes the switch to glycolysis and lactate production by enhancing glycolysis-related genes such as *LDH*, lactate dehydrogenase, and the glucose transporter *GLUT1* (107, 261). *HIF1A* expression has been reported in DCs after

TLR stimulation (261, 262). As the mode of action of MV140 is driven by TLR-mediated signalling, we studied the induction of *HIF1A* at mRNA level. No changes in the expression of *HIF1A* gene were observed after V132 stimulation compared with control cells. However, both MV140- and MV140/V132-activated hmoDCs expressed significantly higher mRNA levels of *HIF1A* than excipients- and V132-treated hmoDCs, without significant differences among them (**Figure 31**).

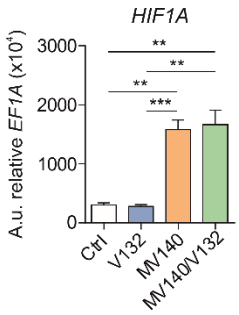

Figure 31. MV140/V132 induces *HIF1A* expression in hmoDCs. Messenger RNA expression levels of *HIF1A* gene in hmoDCs stimulated with control excipients (ctrl), V132, MV140 or MV140/V132 for 18 hours. Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4, with ΔCT defined as the difference between the cycle threshold value for the gene of interest and *EF1A* (n = 5). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05 ***P* < 0.01, and ****P* < 0.001.

The metabolic rewiring towards enhanced glycolysis and lactic fermentation upon MV140/V132 stimulation allows DCs to obtain energy faster and more efficiently for their functional activities (108, 109). Moreover, this reprogramming might be regulated by activation of Akt/mTOR/HIF-1α pathway which is activated (107, 114, 122). Collectively, these results suggested that the metabolic rewiring induced by MV140/V132 might be determined by the contribution of both bacterial components upregulating HIF-1α (261, 262) and *C. albicans* inducing Akt/mTOR signalling pathways (120). This combination might explain the potent glycolytic metabolism in DCs after the treatment with MV140/V132.

The enhanced response and increased metabolic activity are accompanied by epigenetic remodelling (17, 125). In this regard, histone modifications lead to changes in chromatin compaction state, thus upregulating or downregulating gene expression. Some histone methylations are associated with open chromatin, which facilitates the gene transcription. In contrast, others are related with close chromatin and gene repression. To assess the role of histone modifications in the enhanced functional and metabolic response in DCs stimulated with MV140/V132, we performed inhibition experiments using the histone methyltransferase inhibitor 5′-methylthioadenosine (MTA) and the histone demethylase inhibitor pargyline. Interestingly, TNFα, IL-23 and IL-10 production was significantly impaired in the presence of MTA, but preserved in the presence of pargyline after stimulation of hmoDCs with V132, MV140, or MV140/ V132 (**Figure 32A**), suggesting that histone methylation is important for the production of the assayed pro-inflammatory cytokines as well as IL-10 (13, 260). An enhanced IL-10

production was observed in the presence of pargyline compared with stimulated cells without the demethylase inhibitor in all the assayed conditions (**Figure 32A**). Cell viability was not significantly affected in any case and only a slight decrease in cell viability was observed for pargyline after V132 or MV140/V132 stimulation (**Figure 32B**).

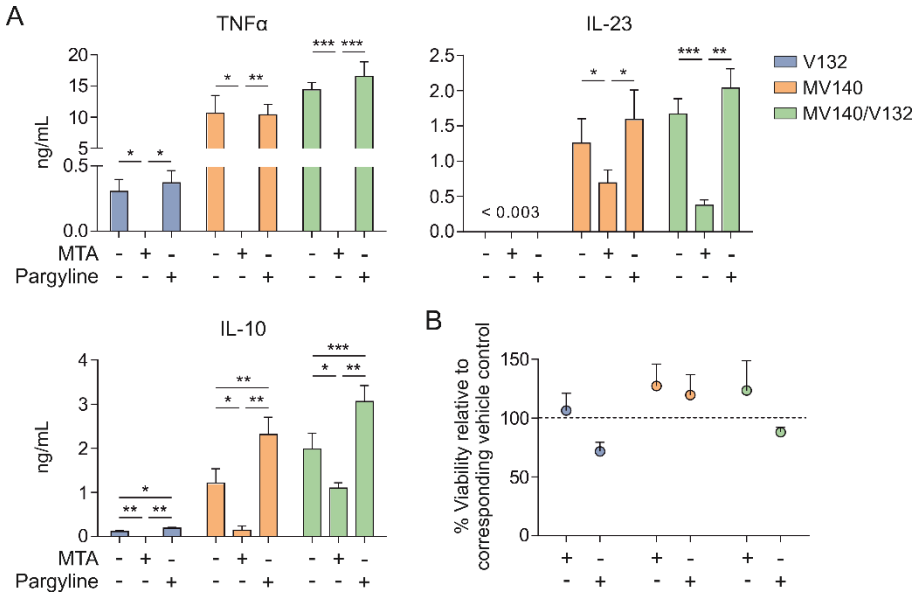

Figure 32. MV140/V132 induces histone methylation of genes involved in TNFα, IL-23 and IL-10 production. (A) Cytokine production by V132-, MV140- and MV140/V132-activated hmoDCs in the presence of 5′-methylthioadenosine (MTA) or pargyline as inhibitors of histone methyltransferases and demethylases, respectively (n = 4-7). (B) Percentage of viability for each assayed condition respect to each vehicle control by using trypan blue exclusion (n = 3-4). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05 ***P* < 0.01, and ****P* < 0.001.

1.5. To study the immunomodulatory properties of MV140/V132 in an *in vivo* mice model

To study the capacity of MV140/V132 to induce *in vivo* systemic responses, in collaboration with Dr. José Luis Subiza and Dr. Carmen M. Díez-Rivero from Inmunotek S.L. (Alcalá de Henares, Spain) and Hospital Clínico San Carlos (Madrid, Spain), BALB/c mice were sublingually immunized with control excipients or MV140/V132 (300 FTU per mL) and the induced T and B cell responses were analysed (**Figure 33**).

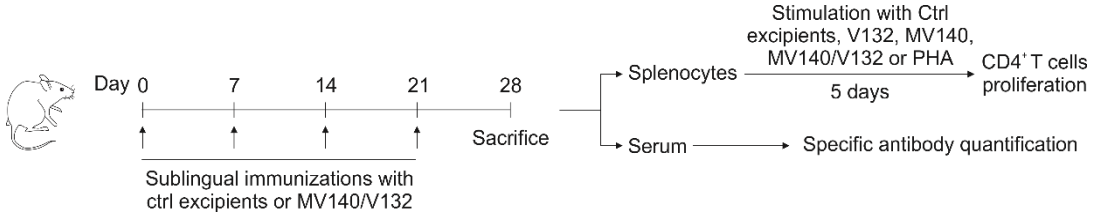

Figure 33. Scheme of the sublingual immunization protocol with control excipients (ctrl) or MV140/V132 and analysis of induced systemic responses. Ctrl, control; PHA, phytohaemagglutinin.

Spleen were collected and *in vitro* stimulated with control excipients, V132, MV140, MV140/V132 and phytohaemagglutinin (PHA) for 5 days to assess the proliferation of splenic CFSE-labeled CD4$^+$ T cells (**Figure 33**). Splenic CD4$^+$ T cells from mice immunized with MV140/V132 or control excipients showed significantly higher proliferation rates after *in vitro* stimulation with all the assayed conditions than after *in vitro* stimulation with control excipients. Remarkably, splenic CD4$^+$ T cells from mice sublingually immunized with MV140/V132 displayed significantly higher proliferation rates than those from control mice after *in vitro* stimulation with not only all the components including in the vaccine (V132 and MV140), but also with the non-related antigen T cell mitogen PHA (**Figure 34**). This result showed an enhanced response induced by MV140/V132 vaccination upon new encounters, which might be mediated by trained immunity mechanisms.

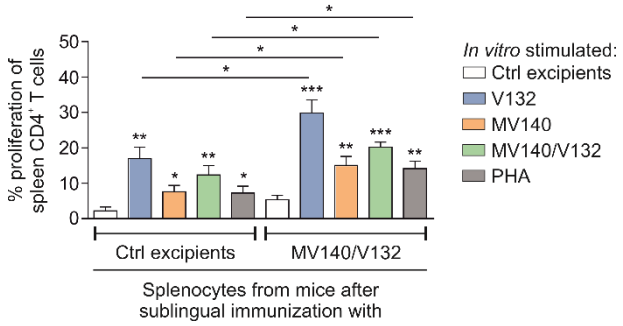

Figure 34. CD4$^+$ T cells from mice sublingually immunized with MV140/V132 displayed significantly higher proliferation rates after *in vitro* stimulation with related and non-related antigens. Proliferation of CFSE labelled CD4$^+$ T cells from splenocytes isolated from mice sublingually immunized with MV140/V132 or control (ctrl) excipients after *in vitro* stimulation with control excipients, V132, MV140, MV140/V132 or phytohaemagglutinin (PHA) for 5 days (n = 7-8 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired Student *t* test. **P* < 0.05 ***P* < 0.01, and ****P* < 0.001.

To evaluate B cell responses in MV140/V132 immunized mice, plasma samples were collected 7 days after the last immunization and serum specific IgG, IgA, IgG1 and IgG2 concentrations were analysed (**Figure 33**). MV140/V132-treated mice displayed significantly higher concentrations of specific IgG and IgA against all the components of the vaccine preparation including *C. albicans* (V132) and the individual bacterial components of MV140 (*K. pneumoniae*, *E. coli*, *P. vulgaris* and *E. faecalis*) than control mice (**Figure 35A**). As shown in **Figure 35B**, the highest specific IgG responses were against the MV140 Gram-negative components *E. coli* and *P. vulgaris* whereas for IgA responses the highest levels were observed for *E. faecalis* and *P. vulgaris*.

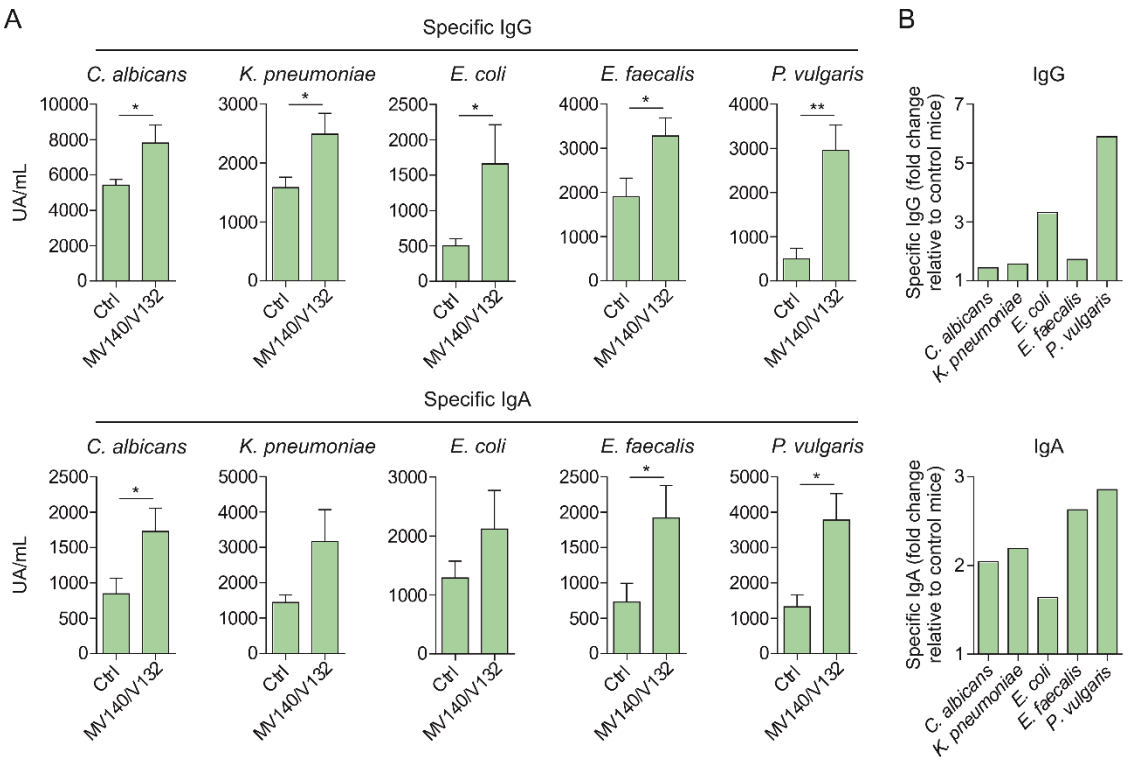

Figure 35. *In vivo* sublingual immunization of mice with MV140/V132 induces specific IgG and IgA antibodies from all the components including in the vaccine formulation. (A) Serum IgG and IgA antibodies specific for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* from mice immunized with MV140/V132 or control excipients (ctrl) (n = 5-6 mice of one independent experiment). (B) Fold change of specific IgG and IgA antibodies generated in mice immunized with MV140/V132 relative to control mice (n = 5-6 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired Student *t* test. **P* < 0.05.

Next, we determined specific IgG1 and IgG2a production. As shown in **Figure 36A**, mice immunized with MV140/V132 produced higher concentrations of IgG1 against all the components contained in the vaccine, only a significant increase of IgG1 against *C. albicans* was observed compared to control mice. In addition, high concentrations of IgG2a against *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* were induced in MV140/V142 immunized mice compared to control-treated mice. Similar IgG1 and IgG2 response was produce against all the stimuli including in the preparation in mice immunized with MV140/V132 (**Figure 36B**). As shown in this figure, similar specific IgG1 and IgG2a responses were induced upon MV140/V132 immunization, suggesting also the priming of IFNγ-producing $CD4^+$ T cells driving IgG2a isotype switch.

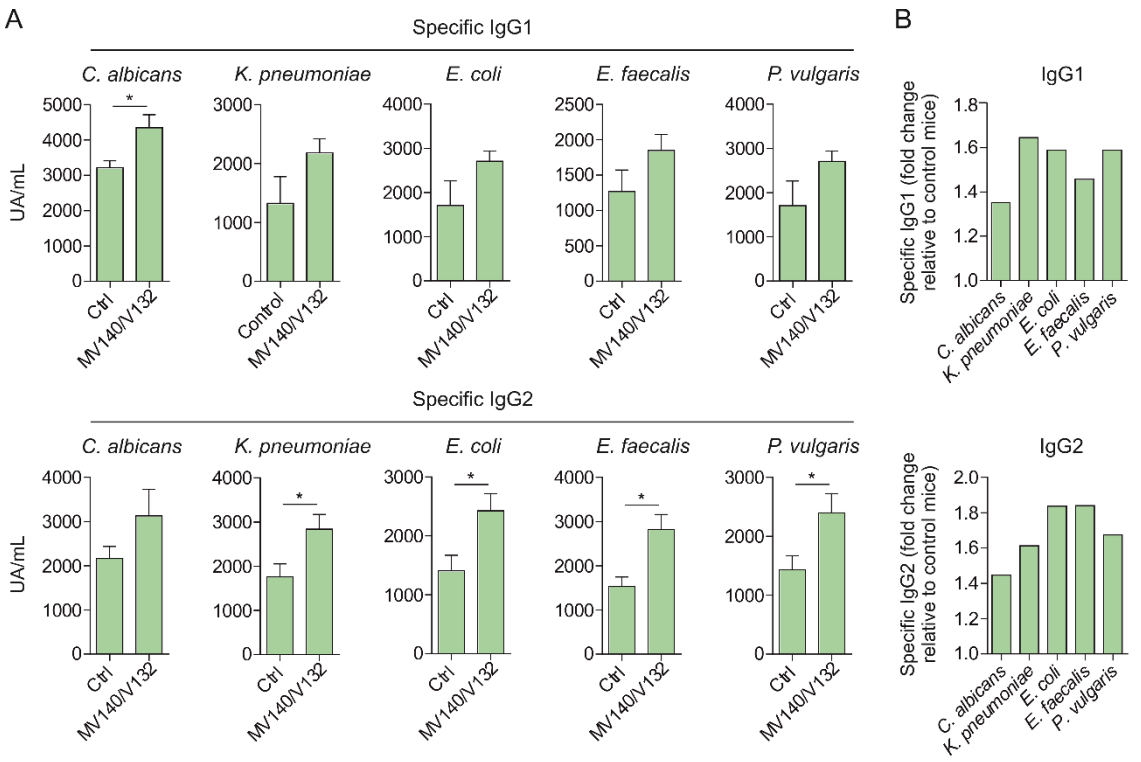

Figure 36. Mice sublingually immunized with MV140/V132 display higher specific IgG1 and IgG2 antibodies against all the components including in the vaccine formulation. (A) Serum IgG1 and IgG2 antibodies specific for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* from mice immunized with MV140/V132 or control excipients (ctrl) (n = 4-5 mice of one independent experiment). (B) Fold change of specific IgG1 and IgG2 antibodies generated in mice immunized with MV140/V132 relative to control mice (n = 4-5 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired Student *t* test. **P* < 0.05.

Then, we wanted to compared the humoral responses induced, previously described in **Figure 35**, with IgG and IgA specific responses in mice upon sublingually immunized with MV140 alone or V132 alone. We observed in **Figure 37A** that mice sublingually immunized with MV140/V132 produced higher IgG responses against *E. coli* and *P. vulgaris* than mice immunized with MV140 alone, whereas only a slight increase for *K. pneumoniae* and a slight decrease for *E. faecalis* were produced. Immunization with MV140/V132 induced higher IgA concentration for *K. pneumoniae* and *P. vulgaris* than immunization with MV140 alone, whereas lower IgA levels were detected for *E. coli* without differences for *E. faecalis* (**Figure 37A**). Remarkably, mice sublingually immunized with MV140/V132 produced higher specific IgG and IgA responses against *C. albicans* than mice treated with V132 alone. This difference was especially pronounced for IgA, which indicates that the incorporation of MV140 into V132 preparations might well significantly promote the production of mucosal IgA antibodies specific for *C. albicans* compared to V132 alone (**Figure 37B**).

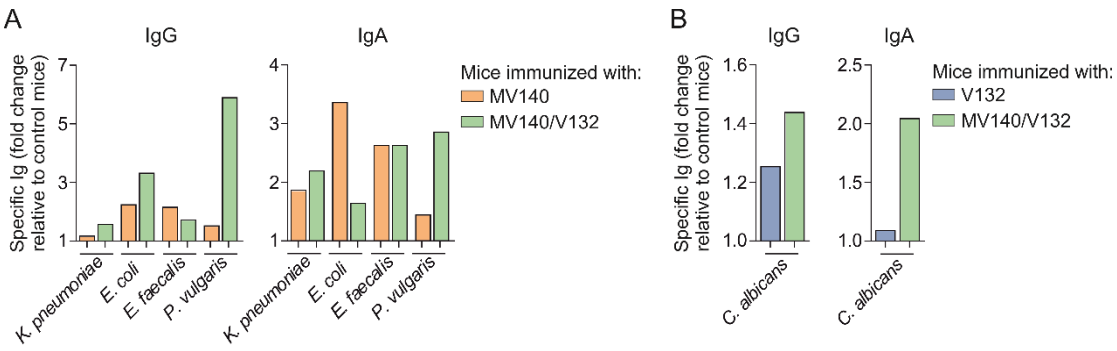

Figure 37. Mice sublingually immunized with MV140/V132 display higher specific IgG1 and IgG2 antibodies against all the components including in the vaccine formulation. (A) Fold change of specific IgG and IgA antibodies for *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* generated in mice immunized with MV140/V132 or MV140 alone relative to control mice (n = 5-6 mice of one independent experiment). (B) Fold change of specific IgG and IgA antibodies for *C. albicans* generated in mice immunized with MV140/V132 or V132 alone relative to control mice (n = 5-6 mice of one independent experiment).

Collectively, our data demonstrated that sublingual immunization of mice with MV140/V132 enhances the proliferation of splenic $CD4^+$ T cells upon secondary *in vitro* exposition to related and non-related antigens. In addition, specific IgG and IgA response against all the components contained in the formulation is potentiated in the presence of both bacterial and fungal components. These results, taken together with the metabolic and epigenetic reprogramming of human DCs, suggested that MV140/V132 might induce trained immunity (10, 263).

Objective 2. Assess the capacity of V132, MV140 and MV140/V132 to induce trained immunity in preclinical human and mice models and elucidation of underlying molecular mechanisms

Trained immunity-based vaccines (TIbV) induce innate immune memory and potent adaptive immune responses against related and non-related pathogens, thus conferring protection against a broader range of pathogens (173). MV140/V132 formulation displays features related to the induction of trained immunity (10, 263). However, it has not explored yet the contribution of each component to these effects. Although, it has been well-described the capacity of different *C. albicans* preparations to confer trained immunity (4), the role of the sublingual vaccine *C. albicans* V132 already used in humans in the context of GUTIs has not been examined yet. In this chapter, we explored the induction of trained immunity in human PBMC and monocytes by V132, MV140 and MV140/V132.

2.1. To study the capacity of V132, MV140 and MV140/V132 to induce trained immunity in human PBMC

We assessed whether V132, MV140 or the simultaneous combination of V132 and MV140 could prime human PBMC to enhance the response after second stimulation. For that, we employed a well stabilised *in vitro* model for the induction of trained immunity (4, 5, 251). PBMC from healthy donors were initially treated with control excipients, V132 alone, MV140 alone or MV140/V132, and the cytokine signature after 24 hours of stimulation was quantified. Next, cells were washed, left resting for 6 days with medium, and then re-stimulated with LPS to assess cytokine production after 24 hours (**Figure 38**).

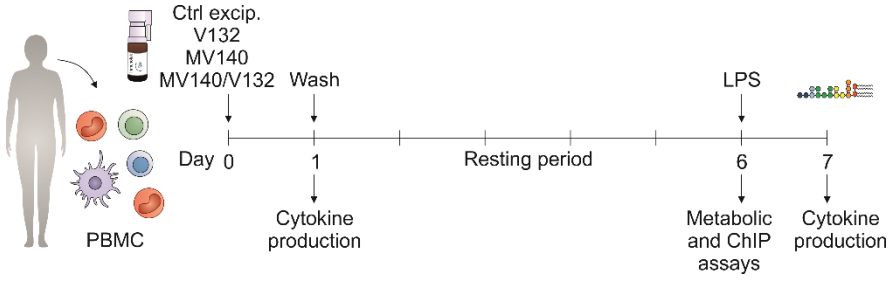

Figure 38. Human *in vitro* model for the induction of trained immunity in human PBMC.

As shown in **Figure 39A**, PBMC stimulation with V132 alone induced significant IL-6 and anti-inflammatory interleukin-1 receptor antagonist (IL-1RA) production. Exposure to MV140 and MV140/V132 significantly increased the production of pro-inflammatory cytokines IL-6, TNFα and IL-1β compared to control excipients or V132.

However, MV140 induced significantly higher concentration of IL-1β than MV140/V132. Although overall anti-inflammatory cytokine IL-10 production was increased after stimulation with MV140 than with control, V132 or MV140/V132, significant differences were not detected. MV140 or MV140/V132-stimulated PBMC also significantly produced higher IL-1RA levels after 24 hours, with higher levels observed after MV140 or MV140/V132 than V132 stimulation (**Figure 39A**). Consequently, the IL-1β/IL-1RA ratio was significantly higher when PBMC were stimulated with MV140 or MV140/V132 than control excipients or V132, and slight but higher with MV140 than with MV140/V132 (**Figure 39B**). Cell viability was not affected in any assayed conditions (**Figure 39C**).

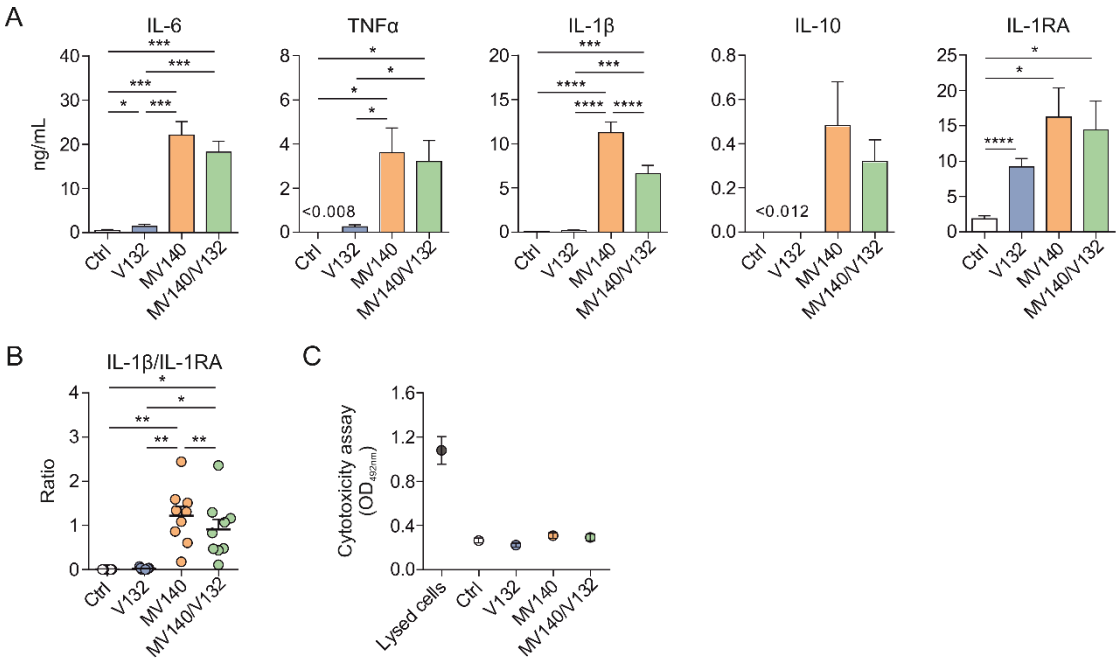

Figure 39. Cytokine signature induced by V132, MV140 or MV140/V132 in human PBMC after 24 hours of stimulation. (A) Cytokine production after stimulation of human PMBC with control excipients (ctrl), V132, MV140 or MV140/V132 for 24 hours in cell-free supernatants (n = 6-11). (B) IL-1β/IL-1RA ratio produced by human PBMC after 24 hours in the indicated conditions (n = 9). (C) Cytotoxicity assay after stimulation of human PBMC for 24 hours. Lysed cells are included as a control (n = 6). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

After 6 days of resting, PBMC were re-stimulated with LPS for 24 hours and cytokine production was quantified. Remarkably, PBMC primed with V132 displayed a trained response on day 7 after re-stimulation with LPS resulting in a significant IL-6 and TNFα production than control excipients, suggesting a trained immunity induction. This effect was not observed when PBMC were stimulated with MV140, and remarkably, not with V132 in the presence of MV140 (MV140/V132) (**Figure 40**). Collectively, these results showed that PBMC upon 24 hours of stimulation display a pro-inflammatory phenotype with an increased pro-inflammatory cytokine, but also IL-1RA, whereas V132-

treated PBMC only produce IL-1RA. In contrast, only V132, but not MV140 or MV140/V132, induces trained immunity in human PBMC.

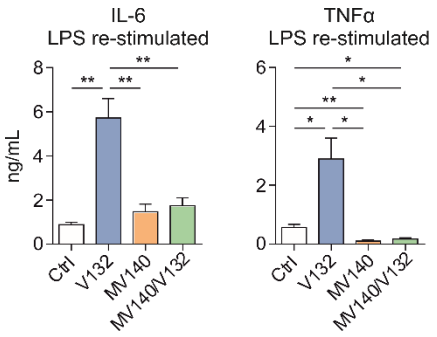

Figure 40. V132 induces trained immunity in human PBMC. PBMC were incubated for 24 hours with control excipients (ctrl), V132, MV140 or MV140/V132. After 6 days, they were re-stimulated with LPS and cytokines were measured 24 hours later (n = 11). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *P < 0.05, and **P < 0.01.

2.2. To study the main metabolic and epigenetic changes promoted by V132 in human PBMC

As mentioned before, metabolic and epigenetic rewiring are two main pillars of trained immunity (17, 263). Therefore, we wanted to investigate two of the main pathways that support the metabolic reprogramming to acquire a trained immunity phenotype: glycolysis and OXPHOS (120-122). For that, we performed the same type of *in vitro* trained immunity experiments. PBMC were treated with control excipients, V132, MV140 or MV140/V132 for 24 hours, washed once and incubated in medium for 6 days when metabolic assays were performed (prior to re-stimulation) (**Figure 38**).

We initially quantified the lactate production in cell-free supernatant. Levels of lactate produced by PBMC primed with V132, MV140 and MV140/V132 were significantly higher after 6 days of resting compared to control cells, reflecting an increase in the activity of glycolysis. Interestingly, PBMC treated with V132 produced a significantly increased in lactate concentrations than cultures with MV140 or MV140/V132 (**Figure 41**).

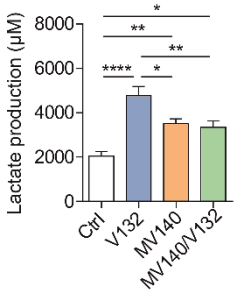

Figure 41. PBMC display an enhanced lactate production after trained with V132. Human PBMC were incubated for 24 hours with control excipients (ctrl), V132, MV140 or MV140/V132. On day 6 (prior to re-stimulation), lactate production was quantified in cell culture supernatants (n = 12). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Then, we performed functional metabolic experiments using a Seahorse bioanalyzer to monitor real-time extracellular acidification rate (ECAR) and mitochondrial oxygen consumption rate (OCR) after 6 days of resting of the initially V132, MV140 or

MV140/V132 exposed PBMC. PBMC trained with V132 showed an increased basal and maximal ECAR, as well as glycolytic reserve compared to PBMC treated with MV140, MV140/V132 or control excipients. Only a slight but significant increase in basal and maximal ECAR was observed after the stimulation with MV140/V132 compared to MV140 or control excipients (**Figure 42A**). Moreover, basal and maximal respiration, ATP production coupled to respiration, and spare respiratory capacity were significantly higher after V132 training than the other assayed stimuli (**Figure 42B**). We demonstrated that trained cells with V132 shift a glucose metabolism towards glycolysis, lactic fermentation and OXPHOS, as previously described for other trained immunity inducers (9, 122).

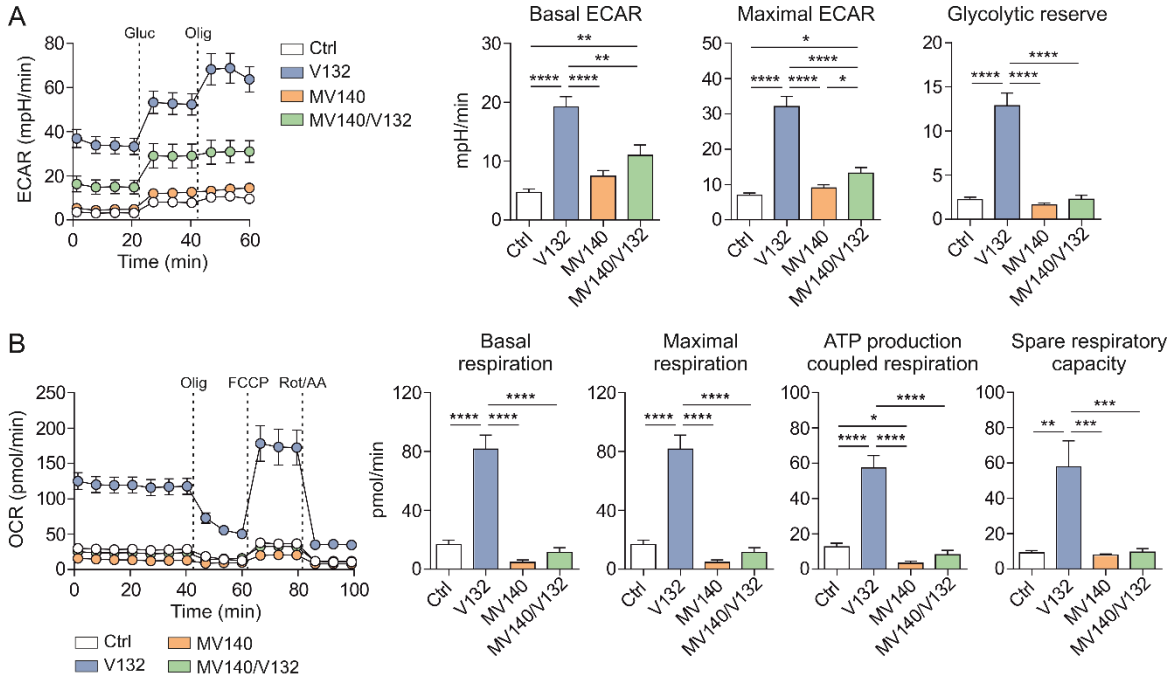

Figure 42. V132-trained cells exhibit a metabolic rewiring with an increased glycolysis and OXPHOS. Human PBMC were incubated for 24 hours with control excipients (ctrl), V132, MV140 or MV140/V132. On day 6 (prior to re-stimulation), metabolic assays were performed. (A) Kinetic study of extracellular acidification rate (ECAR) in control (ctrl)-, V132-, MV140- or MV140/V132-trained PBMC by sequential addition of glucose (Gluc) and oligomycin (Olig) (n = 7 donors, 5 repeated measures of each donor). (B) Kinetic study of mitochondrial oxygen consumption rate (OCR) in ctrl-, V132-, MV140- or MV140/V132-trained PBMC by sequential addition of oligomycin (Olig), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and rotenone/antimycin A (Rot/AA) (n = 7 donors, 5 repeated measures of each donor). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Metabolic changes are directly linked with epigenetic rewiring in trained monocytes (10, 122). To analyses whether the enhanced production of pro-inflammatory cytokines after the second re-stimulation with LPS might be associated to V132-induced epigenetic changes, we performed the same type of *in vitro* trained immunity

experiments (**Figure 38**). We studied chromatin status near the *IL6* and *TNFA* genes by quantifying H3K27ac and H3K9me3 histone marks by chromatin immunoprecipitation (ChIP) analysis in V132-trained PBMC at day 6 (prior to re-stimulation). H3K27ac was previously described as the histone mark associated with open chromatin and active gene transcription in trained immunity, whereas H3K9me3 is considered a repressor mark (9, 10, 130). PBMC that had been trained with V132 PBMC displayed an increase of H3K27ac and a decrease of H3K9me3 at the promoter regions of pro-inflammatory genes *IL6* and *TNFA* (**Figure 43A and B**, respectively), as previously described for other training inducers (18, 122, 131, 264, 265).

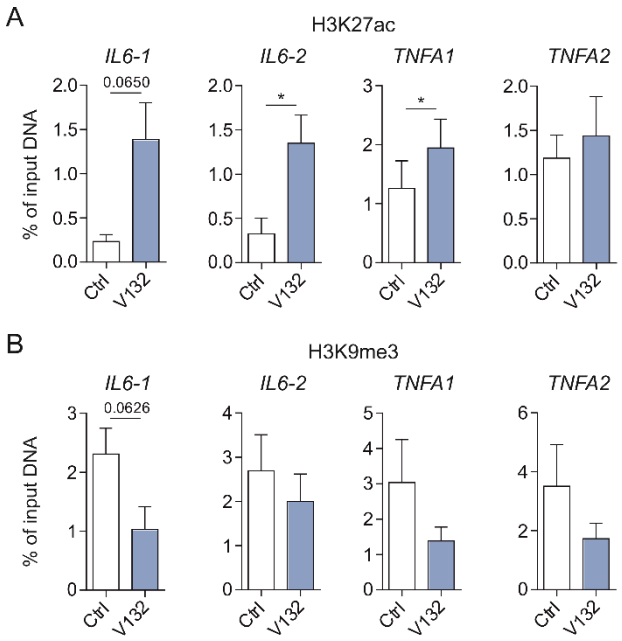

Figure 43. V132-trained cells display epigenetic reprogramming of pro-inflammatory genes. Human PBMC were incubated for 24 hours with control excipients (ctrl), V132, MV140 or MV140/V132. On day 6 (prior to re-stimulation), epigenetic assays were performed. H3K27ac (A) and H3K9me3 (B) histone modifications were determined at the promoter sites of *IL6* and *TNFA* using two different primers for each one (n = 5-6). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05.

2.3. To study the capacity of V132, MV140 and MV140/V132 to induce trained immunity in human monocytes

To further verify whether V132 induces trained immunity in purified human monocytes, the same *in vitro* model for trained immunity was performed using human monocytes isolated form healthy donors (**Figure 44**).

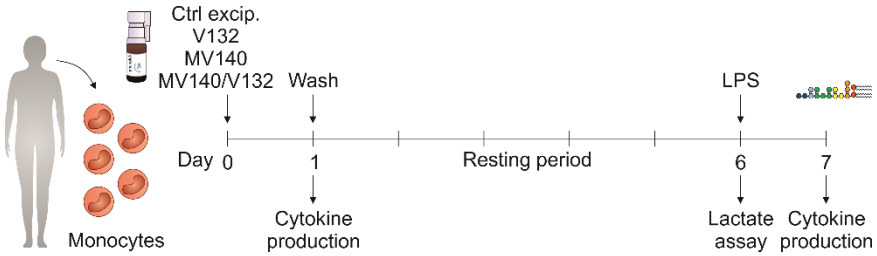

Figure 44. Human *in vitro* model for the induction of trained immunity in human monocytes.

Monocytes were treated with control excipients, V132, MV140 or MV140/V132 for 24 hours to quantified the cytokine production. Initial exposure of monocytes to V132 alone did not induce pro-inflammatory cytokines, in contrast to the IL-6, TNFα and IL-1β production when stimulated with MV140 alone or MV140/V132. Interestingly, monocytes simultaneously activated with MV140/V132 displayed significantly higher levels of TNFα and lower IL-1β than MV140 alone. Stimulation of monocytes with all the assayed conditions increased the concentration of the anti-inflammatory cytokine IL-1RA, which was significantly higher upon V132 treatment than MV140 or MV140/V132 (**Figure 45A**). Therefore, the IL-1β/IL-1RA ratio was significantly increased when monocytes were treated with MV140 or MV140/V132 than with V132 or control excipients. MV140-treated monocytes displayed a higher IL-1β/IL-1RA ratio than cells stimulated with MV140/V132 (**Figure 45B**). Cell viability was not affected in any assayed conditions (**Figure 45C**).

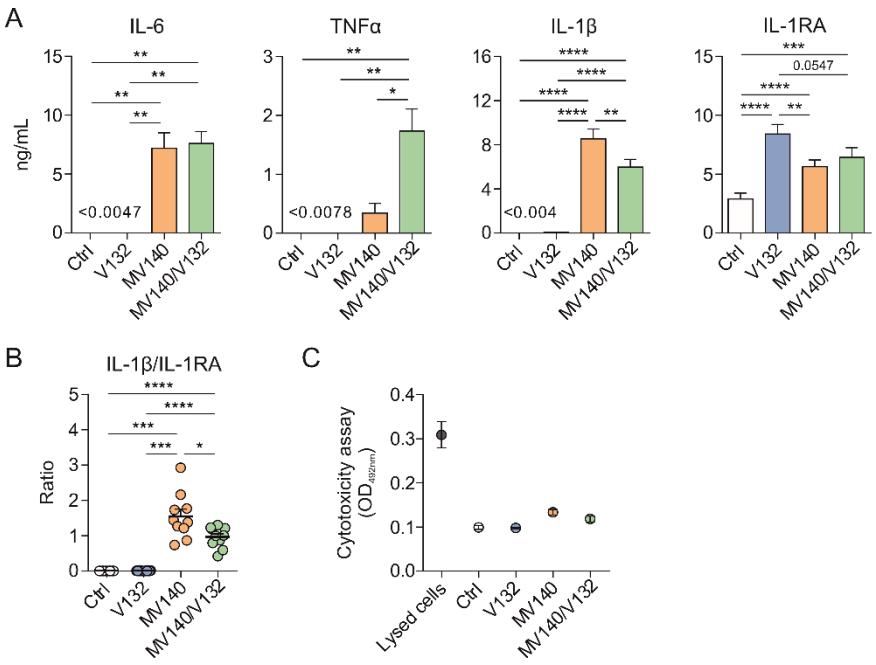

Figure 45. Cytokine signature induced by V132, MV140 or MV140/V132 in human monocytes after 24 hours of stimulation. (A) Cytokine production after stimulation of human monocytes with control excipients (ctrl), V132, MV140 or MV140/V132 for 24 hours in cell-free supernatants (n = 10). (B) IL-1β/IL-1RA ratio produced by human monocytes after 24 hours in the indicated conditions (n = 10). (C) Cytotoxicity assay after stimulation of human monocytes for 24 hours. Lysed cells are included as a control (n = 6). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

We assayed the lactate production in monocytes primed with V132, MV140 or MV140/V132 after 6 days of resting (prior to re-stimulation). Monocytes treated with all the assayed conditions significantly increased the production of lactate compared with control cell, reflecting an enhanced glycolysis metabolism. According to the previous

data with PBMC, monocytes trained with V132 produced higher lactate concentration compared to the other assayed stimuli, without significant differences observed in this case (**Figure 46**).

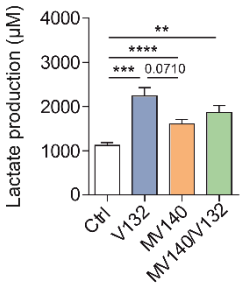

Figure 46. Monocytes display an enhanced lactate production after trained with V132. Human monocytes were incubated for 24 hours with control excipients (ctrl), V132, MV140 or MV140/V132. On day 6 (prior to re-stimulation), lactate production was quantified in cell culture supernatants (n = 11). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Then, monocytes were re-stimulated with LPS for 24 hours after 6 days of resting. As shown in **Figure 47**, V132-trained monocytes produced significantly higher amounts of IL- 6 and TNFα upon re-stimulation 7 days after first priming and resting with LPS, without IL-10 induction. Collectively, these data indicated that in the assayed conditions V132, but not MV140 alone or MV140/V132, induced trained immunity also in purified human monocytes, supporting the previous result in PBMC cultures.

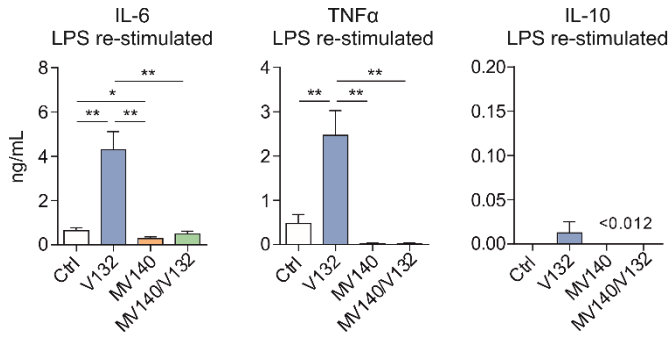

Figure 47. V132-trained monocytes display enhanced innate immune responses upon *in vitro* secondary stimulation with LPS. Monocytes were incubated for 24 hours with control excipients (ctrl), V132, MV140 or MV140/V132. After 6 days, they were re-stimulated with LPS and cytokines were measured 24 hours later (n = 10). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *P < 0.05, and **P < 0.01.

Finally, we explored whether monocytes could display an enhanced response to MV140 used as secondary stimulus when cells were trained with V132. (**Figure 48A**). V132-trained monocytes after MV140 re-stimulation produced significantly higher concentrations of IL-6 and TNFα compared to control monocytes. Remarkably, the production of pro-inflammatory cytokines by V132-trained monocytes was higher upon secondary stimulation with MV140 than LPS (**Figure 48B**).

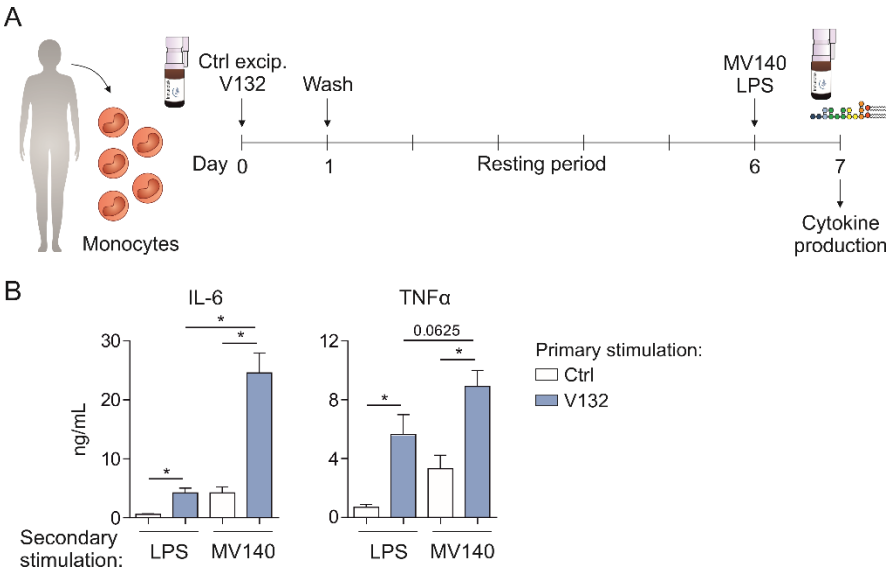

Figure 48. V132-trained monocytes display enhanced innate immune responses upon *in vitro* secondary stimulation with MV140. (A) Human *in vitro* model for the induction of trained immunity in human monocytes. (B) Monocytes were incubated for 24 hours with control excipients (ctrl) or V132 (primary stimulation). On day 7, after 24 hours of re-stimulation with LPS or MV140 (secondary stimulation), IL-6 and TNFα concentration in the supernatants was measured (n = 6). Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. *$P < 0.05$.

Collectively, we reported that initial priming with *C. albicans* V132 induces trained immunity significantly enhancing the capacity of both human monocytes to mount potent pro-inflammatory responses upon a secondary exposure to MV140. Several studies have described the capacity of *C. albicans* and its cell wall component β-glucan as trained immunity inducers (4, 120). After 24 hours of priming with V132, monocytes and PBMC did not produced pro-inflammatory cytokines (only IL-6 was observed in PBMC culture) (4). In addition, V132-treated cells only produced the inhibitory cytokine IL-1RA, as previously reported for other trained immunity inducers (266, 267). IL-1RA binds to the same IL-1β receptor impairing IL-1β-mediated signalling thus regulating the inflammation during infectious processes (268).

On the contrary, first stimulation of monocytes and PBMC with MV140 or MV140/V132 induced a significant increase of pro-inflammatory cytokines as well as IL-1RA concentration. However, when cells were re-stimulated with LPS after 6 days of resting, concentrations of IL-6 and TNFα were very low. It suggested that MV140 and MV140/V132 generate unresponsive monocytes and PBMC to the secondary exposition. Supporting our data, it has been previously reported that TLR signalling through LPS may counteract the trained induction by β-glucan (260), and that LPS induces tolerance in human monocytes, keeping the cells in an unresponsive state to secondary stimulation (123, 264). Due to MV140 is composed of 75% of Gram-negative bacteria containing

LPS, MV140 might also partially reverse V132-induced training of monocytes. The incorporation of MV140 to the V132 formulation allows to include the bacterial antigens contained in MV140 (*K. pneumoniae*, *E. coli*, *P. vulgaris* and *E. faecalis*) to specifically promote adaptive immune responses for this stimuli.

2.4. To study the capacity of V132 to induce trained immunity in an *in vivo* mice model

To assess the *in vivo* relevance of our findings, we studied whether V132 exhibits the previously described capacity to induce trained immunity in an *in vivo* preclinical mouse model. BALB/c mice were immunized by intraperitoneal administration of control excipients or V132 (300 FTU per mL) at day 0 and 3 as shown in **Figure 49**.

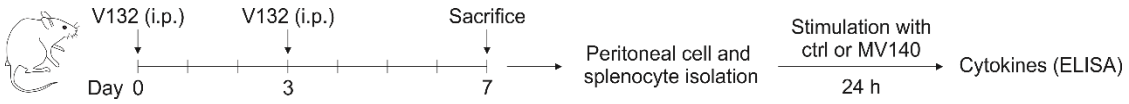

Figure 49. Scheme of the intraperitoneal immunization protocol and analysis of induced systemic response. I.p., intraperitoneal; ctrl, control excipients.

Peritoneal cells and splenocytes were isolated 4 days after the last immunization and cells were *in vitro* stimulated with control excipients or MV140 for 24 hours to assess cytokine production. Intraperitoneal immunization with V132 significantly increased the accumulation of infiltrating cells into the peritoneal cavity compared to control mice (**Figure 50A**).

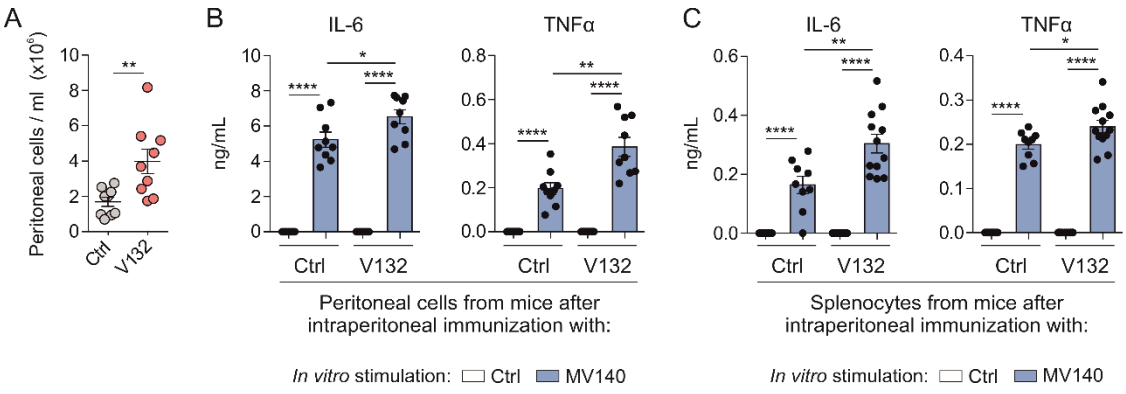

Figure 50. Intraperitoneal immunization of V132 induces trained immunity in mice and enhances innate immune responses after MV140 *in vitro* stimulation. (A) Concentration of peritoneal cells in the peritoneal cavity 4 days after last intraperitoneal immunization (n = 9 mice of two independent experiments). (B) Cytokine production by peritoneal cells from the indicated mice, stimulated *in vitro* with control excipients (ctrl) or MV140 for 24 hours (n = 9 mice of two independent experiments). (C) Cytokine production by splenocytes from the indicated mice, stimulated *in vitro* with ctrl or MV140 for 24 hours (n = 9-12 mice of two independent experiments). Values are mean ± SEM. Statistical significance was determined using Unpaired *t* test. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

Peritoneal cells and splenocytes from mice immunized with both V132 and control excipients responded to MV140 *in vitro* by producing IL-6 and TNFα (**Figure 50B and C**, respectively). Interestingly, cells derived from V132-immunized mice produced significantly higher levels of IL-6 and TNFα than those from mice treated with control excipients (**Figure 50B and C**).

To further investigate the results when V132 was administered through the sublingual route, BALB/c mice were sublingually immunized with control excipients or V132 (300 FTU per mL) for 5 consecutive days during 2 weeks as shown in **Figure 51**.

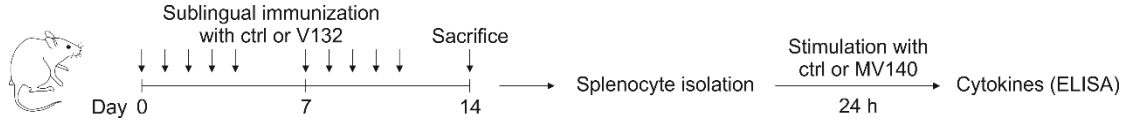

Figure 51. Scheme of the sublingual immunization protocol and analysis of induced systemic response.

Splenocytes were isolated 3 days after the last immunization and cells were *in vitro* stimulated with control excipients or MV140 for 24 hours to quantify cytokine production. Splenocytes from mice sublingually immunized with both V132 and control excipients produced IL-6 and TNFα upon *in vitro* MV140 exposure. Remarkably, the production of TNFα, but not of IL-6, was higher in mice sublingually immunized with V132 than control-treated mice (**Figure 52**).

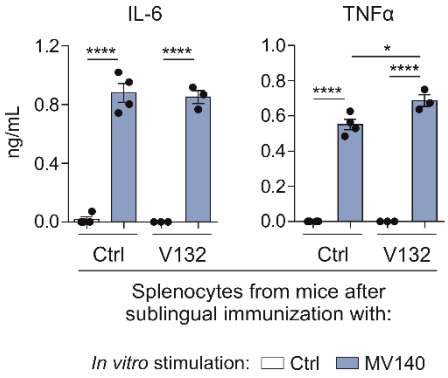

Figure 52. Sublingual immunization of V132 induces trained immunity in mice and enhances innate immune responses after MV140 *in vitro* stimulation. Cytokine production by splenocytes from the indicated mice, stimulated *in vitro* with control (ctrl) or MV140 for 24 hours (n = 3-4 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired *t* test. **P* < 0.05, and *****P* < 0.0001.

Our experiments corroborated the *in vitro* results previously described above for V132 as a trained immunity inducer. *In vivo* immunization via intraperitoneal, but also sublingual with V132 enhanced immune responses when splenocytes were stimulated *in vitro* with MV140. In addition, intraperitoneal administration of V132 was able to promote the recruitment of intraperitoneal cells in the peritoneal cavity. V132-trained peritoneal cells also produced higher pro-inflammatory cytokines when were stimulated *in vitro* with MV140. Therefore, these data suggested that *in vivo* trained immunity induction by V132 might confer heterologous protection, at least, against bacterial

components of MV140 (*K. pneumoniae*, *E. coli*, *P. vulgaris* and *E. faecalis*) with common features to TIbV (18, 19, 173, 177, 269).

Several studies have previously reported that TIbV might also enhance adaptive immune responses against heterologous pathogens (11, 173). Thus, we assessed the potential effects of V132 training on adaptive responses. For that, BALB/c mice were previously immunized with sublingual control excipients or V132 (300 FTU per mL) for 5 consecutive days during 2 weeks and then, 3 days after the last immunization, mice were treated *in vivo* with MV140 (10^9 bact. per mL). Peritoneal cells and splenocytes were isolated after 7 days of MV140 administration (**Figure 53**).

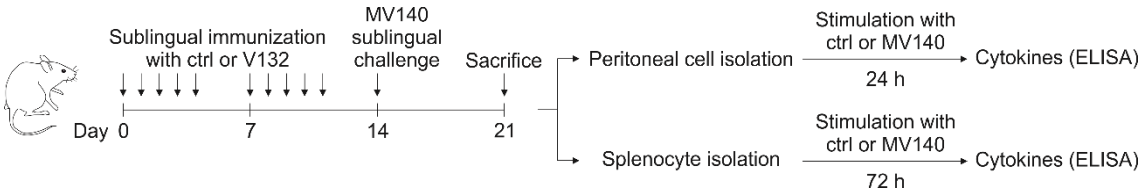

Figure 53. Scheme of the sublingual immunization protocol and analysis of induced systemic response.

As shown in **Figure 54A**, sublingual immunization with V132 and subsequent administration of MV140 induced a recruitment of peritoneal cells thus significantly increasing the cell infiltration into the peritoneal cavity. Peritoneal cells from both mice groups stimulated *in vitro* with MV140 displayed a significant increase in IL-6 and TNFα production than those without MV140 stimulation. In addition, the levels of TNFα, but not of IL-6, produced by peritoneal cells from mice sublingually immunized with V132 were slightly and significantly higher than those induced by peritoneal cells from control mice upon *in vitro* treatment with MV140 (**Figure 54B**).

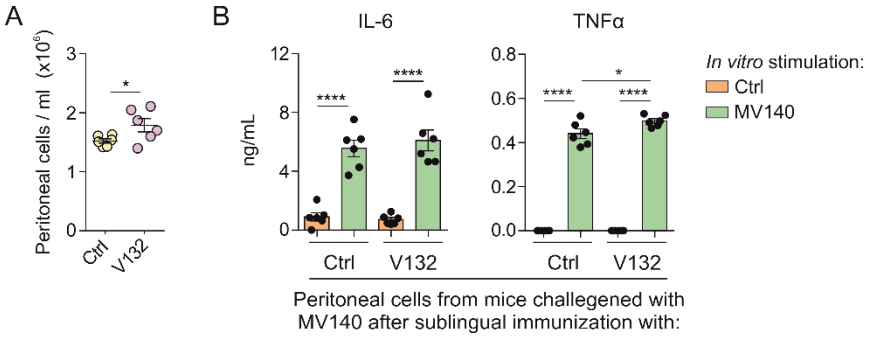

Figure 54. Sublingual immunization of V132 and subsequent treatment with MV140 enhances innate immune responses in peritoneal cells after MV140 *in vitro* stimulation. (A) Concentration of peritoneal cells in the peritoneal cavity 7 days after MV140 administration (n = 6 mice of one independent experiment). (B) Cytokine production by peritoneal cells from the indicated mice, stimulated *in vitro* with control excipients (ctrl) or MV140 for 24 hours (n = 6 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired *t* test. **P* < 0.05, and *****P* < 0.0001.

Then, we assessed whether V132 potentiates specific T cell responses to MV140, splenocytes from immunized mice were stimulated *in vitro* with control excipients or MV140 for 72 hours (**Figure 53**). Mice immunized sublingually with V132 and control mice, and then, subsequent administration of MV140 produced a significant IL-17A, IFNγ and IL-10 *in vitro* response to upon MV140 stimulation. Remarkably, the IL-17A production, but not IFNγ or IL-10, was significantly higher in splenocytes from V132-immunized mice after *in vitro* MV140 stimulation than the concentration induced by splenocytes from control mice (**Figure 55**), suggesting the potential immunomodulatory capacity of sublingual V132 vaccination to potentiate Th17 adaptive immune responses in mice against MV140.

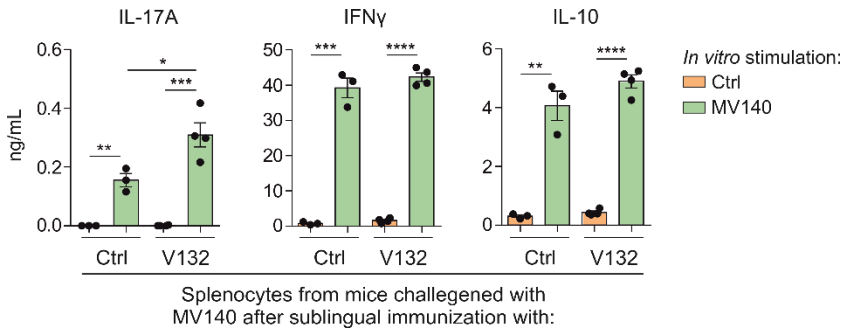

Figure 55. Sublingual immunization of V132 and subsequent treatment with MV140 enhances adaptive immune responses against MV140. Cytokine production by splenocytes from the indicated mice, stimulated *in vitro* with control excipients (ctrl) or MV140 for 72 hours (n = 3-4 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Collectively, these data demonstrated that V132 induces *in vitro* and *in vivo* trained immunity significantly enhancing innate and adaptive immune responses triggered by MV140. Moreover, the induction of training relies on metabolic and epigenetic reprogramming to adapt functional activities of trained cells. We have reported in human and mice preclinical models that V132 might be used as a sublingual vaccine for the prevention of RVVCs. Moreover, due to the association with RUTIs (137, 160, 161), prior vaccination with V132 could potentiate MV140 effects for the prevention in patients where both infections coexist (135).

In the first chapter we have demonstrated that vaccination with the combination of both MV140 and V132 in a single preparation potentiated specific adaptive immunity against the components included in the vaccine and unrelated antigens. Here, we uncovered that V132-induced trained immunity enhances immune response of MV140. However, more detailed studies are necessary to further confirm whether the immune response against pathogens is more robust after sequential administration of

MV140/V132 or with two doses for each individual vaccine. In summary, these results might well contribute to the development of novel therapeutic alternatives to antibiotics and antifungal treatment for recurrent GUTIs.

Block B: Study of the molecular mechanisms by which the tumoral carbohydrate Ca10 immunomodulates human DCs

Objective 3. Study of the capacity of the carbohydrate Ca10 to induce tolerogenic human DCs and the elucidation of underlying molecular mechanisms

DCs infiltrating tumors recognize antigens, which are then processed and presented to naïve T cells to initiate proper adaptive immune responses. The ability of DCs to generate suppressive Treg cells is strongly influenced by exogenous signals encountered in the tumor microenvironment (TME) (21). Tumor progression is associated with alterations in glylcoconjugates located on cancer cell surface having a strong influence on tumor escape, angiogenesis and metastases (231, 234). In addition, tumor-associated carbohydrates inhibit immune responses against the tumor through the generation of Treg cells, among other mechanisms (237-239). In this context, the carbohydrate A10 (Ca10) initially described in the Ehrlich tumor (ET) cells and in certain human adenocarcinoma, might be involved in tumor progression (244, 245, 250). However, the potential functional implications of this tumoral carbohydrate in cancer remain largely unknown. In this chapter, we studied how this tumor-derived carbohydrate Ca10 might condition the capacity of DCs to polarize functional Treg cells and the underlying mechanisms involved in such effects.

3.1. To assess the potential relationship between circulating Ca10, tumor size and Treg cell generation in a solid Ehrlich tumor-bearing mice model

ET is a paradigmatic example of tumor progression across different antigenic backgrounds, representing a suitable model to unravel tumor escape mechanisms (246, 247). It was described that Ca10 is spontaneously released from ET cells and can be detected in the serum of solid ET-bearing mice (245). Therefore, we wanted to assess the potential relationship between tumor growth, circulating Ca10 in serum and Treg cell generation as a possible mechanism of tumor escape in a solid ET-bearing mice model. In collaboration with Dr. José Luis Subiza and Dr. Marcos Viñuela from Inmunotek S.L. (Alcalá de Henares, Spain) and Hospital Clínico San Carlos (Madrid, Spain), C57BL/6J mice were inoculated with ET cells and were sacrificed at different days (22, 32, 42 and 58 days) of tumor development. Then, tumor size and levels of Ca10 in serum were quantified and the induction of Treg cells was analysed in freshly isolated spleens (**Figure 56**).

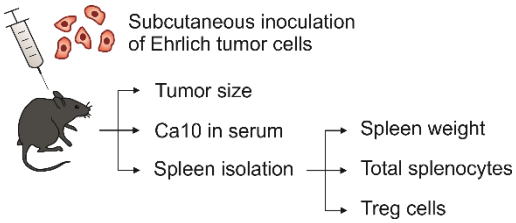

Figure 56. Inoculation of Ehrlich tumor (ET) cells in mice and experimental overview. ET cells were inoculated in mice by subcutaneous injection into the left groin. After 22, 32, 42 or 58 days of ET cell inoculation, mice were sacrificed to monitor the tumor size. Spleens and blood were collected to assess Treg cell generation and circulating Ca10, respectively.

A strong positive correlation was observed between serum Ca10 levels and tumor size (**Figure 57A**). The percentage of splenic FOXP3$^+$ Treg cells was also positively correlated with both serum Ca10 levels and tumor size (**Figure 57B**). Consistent with previous studies, a strong splenomegaly related to tumor size was observed concomitant with a positive correlation between total number of splenocytes and tumor size (**Figure 57C**) (270, 271).

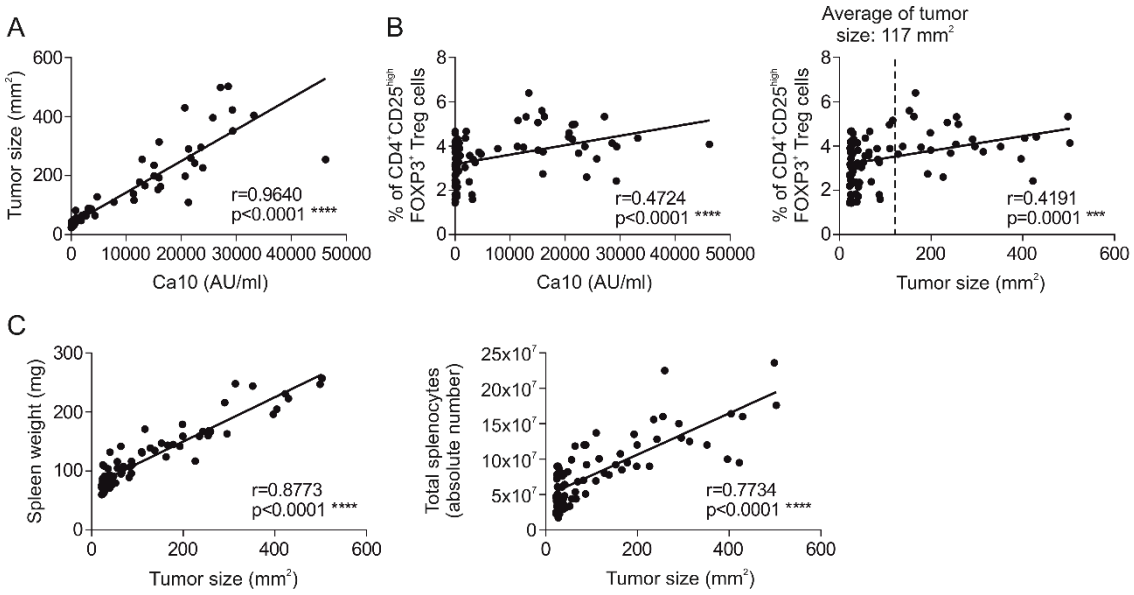

Figure 57. Correlations in mice bearing ET. (A, B) Correlation of serum Ca10 levels with tumor size (A) and percentage of splenic Treg cells with serum Ca10 levels or tumor size (B) in ET-bearing mice (n = 79 mice of two independent experiments). (C) Correlation of spleen weight or total number of cells in spleen with tumor size in ET-bearing mice (n = 79 mice of two independent experiments). Values are mean ± SEM. Statistical significance was determined using Spearman test. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

The variability of tumor size allowed the setting of a cut-off point to stratify mice bearing large (above the average, >117 mm²) and small (below the average, <117 mm²) tumors. The percentage of splenic FOXP3$^+$ Treg cells was significantly higher in mice bearing large than small tumors independently of the time of tumor evolution. Remarkably, mice bearing tumors always displayed significantly higher percentage of Treg cells than controls (**Figure 58**).

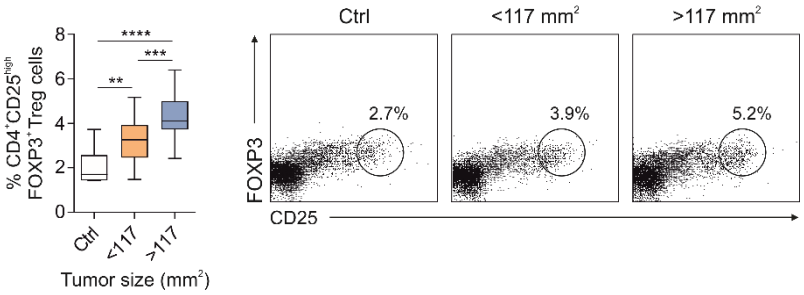

Figure 58. Induction of Treg cells in mice bearing ET. Percentage of splenic $CD4^+CD25^{high}FOXP3^+$ Treg cells from no tumor-bearing mice (ctrl) and mice with small (<117 mm^2) or large (>117 mm^2) tumors. The average of all tumor size was used as cut off (117 mm^2). Representative dot plots of intranuclear FOXP3 staining are shown (n = 6 mice for ctrl, n = 47 and 26 mice for small and large tumors, respectively, of two independent experiments). Values are mean ± SEM. Statistical significance was determined using Mann-Whitney test. $^{**}P < 0.01$, and $^{****}P < 0.0001$.

Then we studied the relationship between the tumor growth at different days of ET cell inoculation with the induction of splenic Treg cells. Mice sacrificed at different time were also stratified in mice bearing large (>117 mm²) and small (<117 mm²) tumors and then, Treg cell generation was studied up to 24 and from 48 days after tumor inoculation. When considering the time of tumor evolution, the percentage of splenic Treg cells from mice bearing large tumors (>117 mm^2) developed at earlier time points (up to 24 days after inoculation of ET cells) was significantly higher than in large tumors reaching the final size (>117 mm^2) at later time points (from 48 days onwards after inoculation of ET cells) without differences observed among small tumors collected at different time points after inoculation. The percentage of Treg cells was always significantly higher in ET-bearing mice than controls and, independently of the time of tumor evolution, Treg cell frequency in spleen was significantly higher in mice bearing large than small tumors (**Figure 59**).

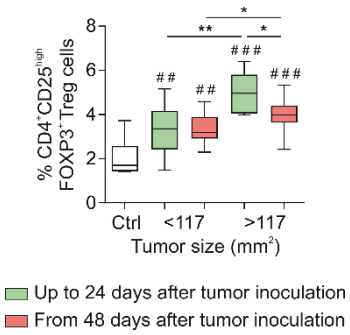

Figure 59. Induction of Treg cells in mice bearing ET. Percentage of splenic $CD4^+CD25^{high}FOXP3^+$ Treg cells from no tumor bearing mice (ctrl) and small or large tumor-bearing mice. Mice were sacrificed up to 24 days after tumor inoculation (green) or from 48 days after tumor inoculation (red) (n = 6 mice for ctrl, n = 30 mice with small tumor sacrificed up to 24 days after ET inoculation, n = 18 mice with small tumor sacrificed from 48 days after ET inoculation, n = 6 mice with large tumor sacrificed up to 24 days after ET inoculation, and n = 19 mice with large tumor sacrificed from 48 days after ET inoculation of two independent experiments). # tumor-bearing mice vs control, * tumor condition vs tumor condition. Values are mean ± SEM. Statistical significance was determined using Mann-Whitney test. $^{**}P < 0.01$, and $^{****}P < 0.0001$.

Collectively, these data strongly suggested a close relationship between serum Ca10 levels, the induction of splenic Treg cells and tumor progression. It was described that Ca10 is spontaneously released from ET cells, which might represent a mechanism of tumor escape (245). Additionally, the positive correlation between the serum levels of Ca10, tumor size and splenic Treg cell frequency suggested that this tumoral carbohydrate could play a role in the generation of Treg cells, thus promoting tumor development and progression.

Then, we further explore whether the carbohydrate Ca10 was directly involved in the generation of Treg cells. The monoclonal antibody A10 was developed by Inmunotek S.L. against the carbohydrate Ca10 inhibiting the ET growth (244, 245, 248). Therefore, we also wanted to assess whether the antibody A10 was able to impair the effect of Ca10 in Treg cell induction. For that, Ca10 was administered to C57BL/6J mice in the presence or absence of antibody A10 for 7 days and sacrificed the next day after the last administration. Then, spleens and blood were obtained to quantify the generation of Treg cells and circulating Ca10, respectively (**Figure 60**).

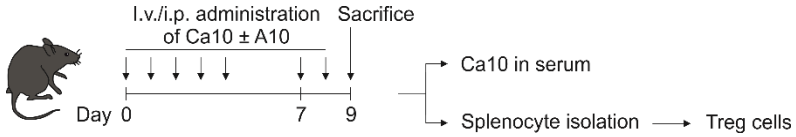

Figure 60. Scheme of the administration protocol with Ca10 in the presence or absence of A10 in mice. Mice were alternatively administered intravenously (i.v.) or intraperitoneally (i.p.) with Ca10 in the presence or absence of antibody A10 for 7 days, and sacrificed the next day after the last inoculation. Spleens and blood were collected to assess Treg cells and circulating Ca10, respectively.

The percentage of splenic FOXP3$^+$ Treg cells was significantly higher in mice administered with Ca10 compared to control mice. Mice administered with Ca10 in the presence of antibody A10 displayed a slightly lower frequency of Treg cells without significant differences compared to Ca10-administered mice, suggesting that the monoclonal antibody might be partially impairing the Treg cell generation promoted by Ca10 (**Figure 61A**). In addition, we can observe a strong positive correlation between serum Ca10 levels and the percentage of splenic FOXP3$^+$ Treg cells (**Figure 61B**).

In summary, these results demonstrated that the carbohydrate Ca10 promotes the generation of splenic Treg cells, which correlated with Ca10 concentration in serum. In addition, ET might be promoting the tumor escape by shedding Ca10 and this carbohydrate could be involved in tumor progression by mechanisms that rely on the generation of functional Treg cells.

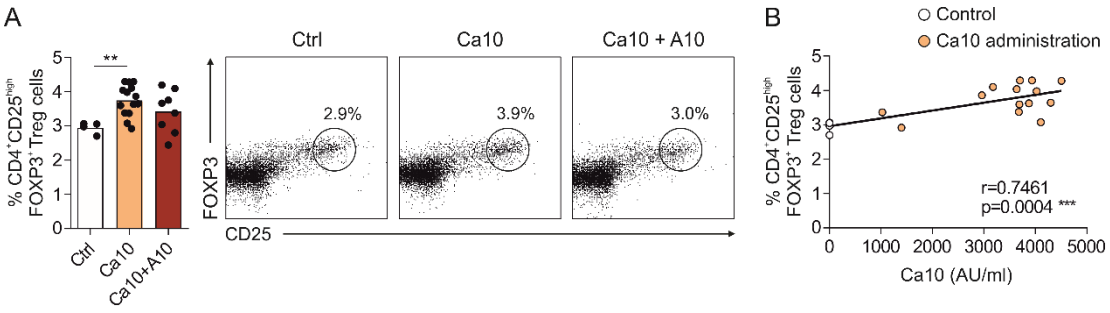

Figure 61. Induction of Treg cells in mice administered with Ca10. (A) Percentage of splenic $CD4^+CD25^{high}FOXP3^+$ Treg cells from control (ctrl) mice and mice that were administered with Ca10 in the presence or absence of the antibody A10. Representative dot plots of intranuclear FOXP3 staining are shown (n = 4 mice for ctrl, n = 14 mice for Ca10 and n = 8 mice for Ca10 in the presence of A10 of one independent experiment). (B) Correlation of percentage of splenic Treg cells with serum Ca10 levels in control or Ca10-administered mice (n = 18 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Mann-Whitney (A) or Pearson (B) test. ***P* < 0.01.

3.2. To study the capacity of the tumoral carbohydrate Ca10 to regulate the phenotype and function of human DCs

DCs play a key role in the generation of Treg cells, which is strongly influenced by tumor antigens and exogenous signals encountered in the TME (21, 37). To further explore the mechanisms involved in the potential capacity of tumoral Ca10 to promote Treg cell generation, we initially investigated the ability of this murine carbohydrate-bearing macromolecule to regulate the phenotype and function of human DCs. HmoDCs were generated as previously described in Materials and Methods and treated with Ca10 or medium (as negative control). After 18 hours of stimulation, activation and functional marker expression, cytokine production and gene expression were analysed. Ca10 stimulation of hmoDCs significantly increased the expression of HLA-DR, CD86/CD83 and the tolerogenic marker PD-L1 (**Figure 62**). PD-L1, which is associated with the capacity of DCs to polarize Treg cells, is a key molecule to impair anti-tumor immune response (84, 272, 273).

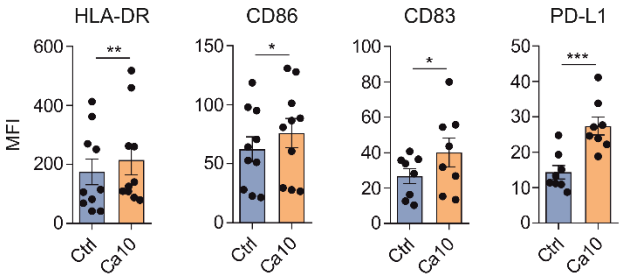

Figure 62. Ca10 induces the expression of costimulatory molecules and tolerogenic marker PD-L1 in hmoDCs. Mean fluorescence intensity (MFI) values for the indicated surface markers after stimulation of hmoDCs with medium (ctrl) or Ca10 for 18 hours (n = 8-10). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Additionally, Ca10-stimulated hmoDCs also produced significantly higher levels of TNFα, IL-6, IL-1β as well as the anti-inflammatory cytokine IL-10 than unstimulated cells (**Figure 63**).

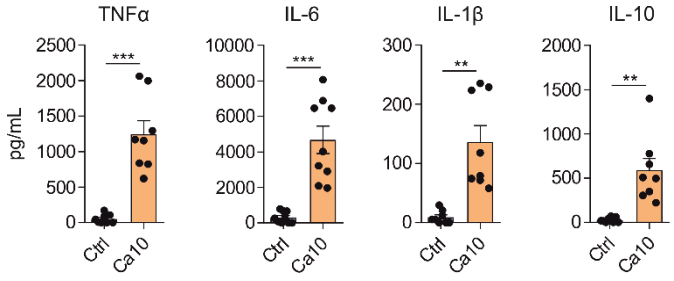

Figure 63. HmoDCs activated with Ca10 produce higher concentrations of pro- and anti-inflammatory cytokines. Cytokine production after stimulation of hmoDCs with medium (ctrl) or Ca10 for 18 hours quantified in cell-free supernatants (n = 8-9). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. ***P* < 0.01, and ****P* < 0.001.

Then, hmoDCs were stimulated with Ca10 for different times to assess the kinetics of cytokine and tolerogenic molecule expression at mRNA level. Kinetic experiments showed that Ca10 rapidly increased the mRNA expression of pro-inflammatory cytokines IL-6 and IL-1β in a time-dependent manner, which declined to almost basal levels after 24 hours of stimulation (**Figure 64A**). In contrast, the mRNA levels of *IL10* (**Figure 64A**) and the tolerogenic molecules *PDL1*, *indoleamine 2,3-dioxygenase* (*IDO*), *suppressor of cytokine signalling* (*SOCS*) *1* and *SOCS3* (**Figure 64B**) remained upregulated after 24 hours of stimulation. However, after Ca10 stimulation the expression of *RALDH1* and *RALDH2*, involved in retinoic acid metabolism, was lower in hmoDCs compared to control cells (**Figure 64B**) (274).

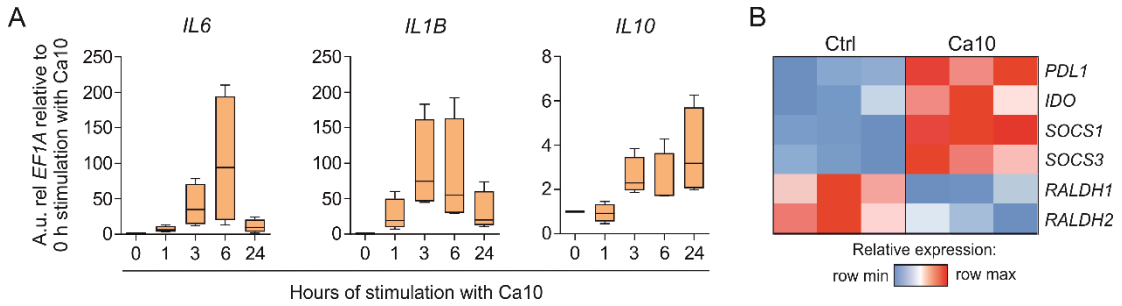

Figure 64. Ca10 promotes the expression of tolerogenic molecules in hmoDCs. (A) Messenger RNA expression levels of *IL6*, *IL1B* and *IL10* genes in hmoDCs treated with Ca10 for 0, 1, 3, 6 and 24 hours relative to Ca10 stimulation at 0 hour. Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4, with ΔCT defined as the difference between the cycle threshold value for the gene of interest and *EF1A* (n = 4). (B) Heatmap representation for gene expression on hmoDCs unstimulated (ctrl) or stimulated with Ca10 for 24 hours (n = 3). Values are mean ± SEM.

IDO expression is upregulated in tumor associated-DCs (86). The enzyme IDO metabolizes tryptophan, which is involved in effector T cell responses, therefore increased IDO expression favour the generation of Treg cells (70, 86). SOCS controls cytokine signalling avoiding excessive immune system activation (275).

Collectively, these data suggested that Ca10 might well imprint tolerogenic features in human DCs (38, 73). All these molecules are known to play a critical role in the induction of regulatory T cells (99).

Supporting these data, blocking of IL-10 significantly enhanced the production of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β, whereas treatment with 1-methyl tryptophan (1-MT), a selective inhibitor of IDO activity, significantly reduced the production of IL-10 in Ca10-stimulated hmoDCs (**Figure 65A**). Blocking of IL-10 or IDO also reduced the induction of PD-L1 in Ca10-stimulated hmoDCs (**Figure 65B**), indicating that the tolerogenic features imprinted by Ca10 in human DCs are partially depending on IL-10 and IDO induction.

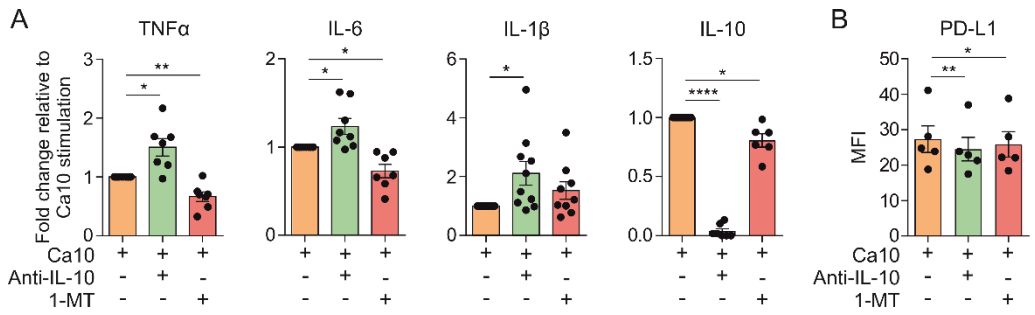

Figure 65. Contribution of IL-10 and IDO in cytokine production and PD-L1 expression induced by Ca10 in hmoDCs. (A) Cytokine production of hmoDCs stimulated with Ca10 in the presence of IL-10 blocking antibody or IDO inhibitor (1-MT) for 18 hours relative to Ca10 stimulation (n = 6-10). (B) Mean fluorescence intensity (MFI) for the expression of surface marker PD-L1 on hmoDCs after stimulation with Ca10 in the presence of IL-10 blocking antibody or 1-MT for 18 hours (n = 5). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001.

3.3. To study the main immune signalling pathways involved in the immunomodulatory effects of the carbohydrate Ca10 in human DCs

DCs are equipped with different Syk-coupled CLRs to sense sugar structures (52, 53). To gain further insights into the molecular mechanisms involved in the mode of action of Ca10 in human DCs, we assessed the capacity of this carbohydrate to activate classical immune pathways by western blot analyses and by inhibition experiments. For western blot experiments, hmoDCs were stimulated with Ca10 or medium for 30 minutes. Ca10 induced a rapid activation of the MAPKs ERK, p38 and JNK and NF-κB as determined

by the increase of the phosphorylated forms of the assayed MAPKs and IκBα, the inhibitor of NF-κB (**Figure 66**).

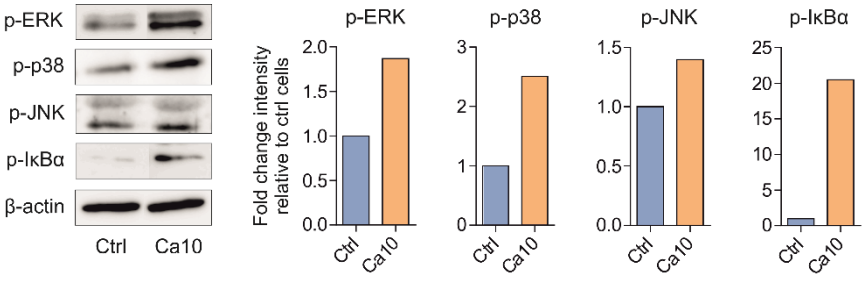

Figure 66. Ca10 activates MAPKs and NF-κB signalling pathways in hmoDCs. Western blot analysis of protein extracts from hmoDCs stimulated with medium (ctrl) or Ca10 for 30 minutes (n = 2-4). Quantification of the reactive phosphorylated bands by scanning densitometry. β-actin was used as a loading control. One representative example is shown.

Then, we used different inhibitors to analyse the contribution of the signalling pathways described above in the cytokine production induced by Ca10 in human DCs. Supporting the previous data, the pharmacological inhibitors U0126 (specific for MEK 1/2, upstream of ERK), SB202190 (specific for p38), SP600125 (specific for JNK) and BAY117082 (specific for NF-κB) significantly inhibited IL-6 and IL-10 production in Ca10-activated hmoDCs. IL-6 and IL-10 production induced by Ca10 stimulation was significantly impaired when DCs were preincubated with EDTA (chelating agent of calcium) and piceatannol (Syk inhibitor) (**Figure 67**).

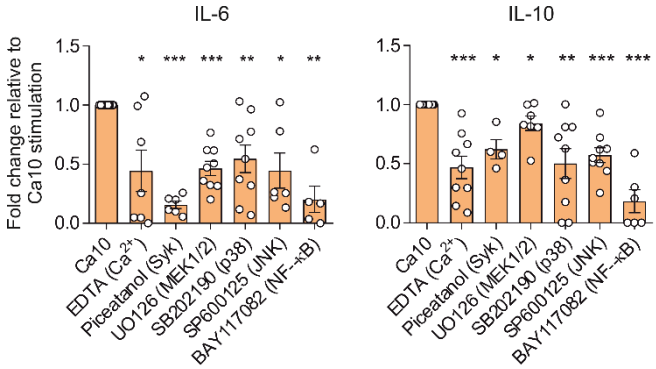

Figure 67. Contribution of Syk-couple CLRs, MAPKs and NF-κB pathways in IL-6 and IL-10 production induced by Ca10 in hmoDCs. Cytokine production of hmoDCs stimulated with Ca10 in the presence of the indicated inhibitors for 18 hours relative to Ca10 stimulation (n = 4-9). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

In summary, these results demonstrated that Ca10 induces the activation of MAPKs- and NF-κB-mediated signalling pathways that regulate the imprinted cytokine signature in hmoDCs. Upon activation, CLRs induces several downstream signalling

pathways, including MAPKs and NF-κB, which under certain circumstances might promote tolerogenic DCs (38, 49, 53, 81). At this regard, our findings suggested that Ca10 might well trigger Syk-couple CLRs expressed on human DCs in a Ca^{2+}-dependent manner, which in turns would initiate the above mentioned downstream signalling pathways imprinting the reported tolerogenic features (**Figure 68**).

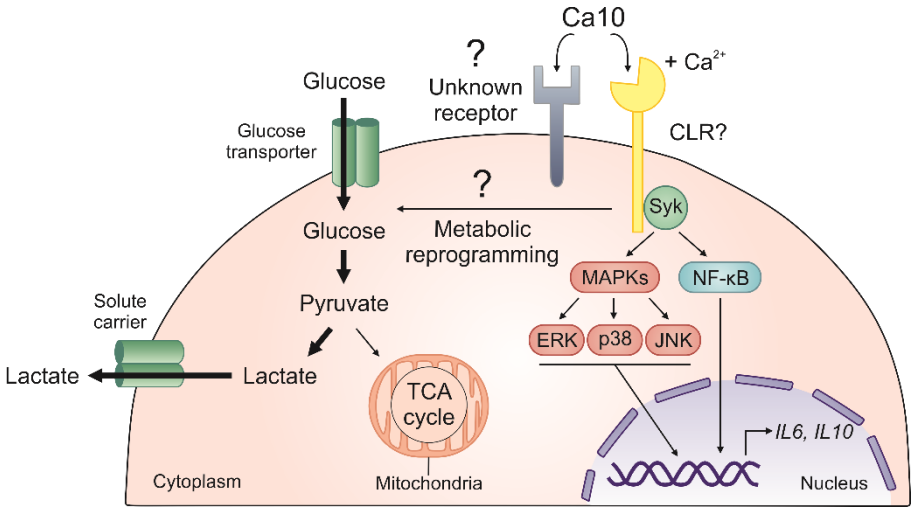

Figure 68. Ca10 activates immune pathways and metabolic reprogramming in hmoDCs. Ca10 might well target Syk-couple CLRs in a calcium dependent manner leading to the activation of MAPKs and NF-κB as the main drivers of the induced cytokine signature in hmoDCs. Ca10 enhances glycolysis and lactic fermentation without affecting mitochondrial oxidative phosphorylation in hmoDCs.

3.4. To study the metabolic changes and their contribution to the tolerogenic properties exerted by Ca10 in human DCs

As previously described, metabolic reprogramming plays a key role in the control of the functional features of DCs by regulating tolerogenicity vs immunogenicity (107, 108, 110). To study the capacity of Ca10 to promote metabolic reprogramming, hmoDCs were stimulated with Ca10 for 18 hours. We performed real-time ECAR, mitochondrial OCR, and glycolytic proton efflux rate (glycoPER) dynamic experiments using a Seahorse bioanalyzer. Basal glycolysis and compensatory glycolysis were significantly increased in Ca10-stimulated human DCs compared to unstimulated cells. In addition, Ca10 did not induce differences in OCR levels compared to medium treatment in hmoDCs (**Figure 69**).

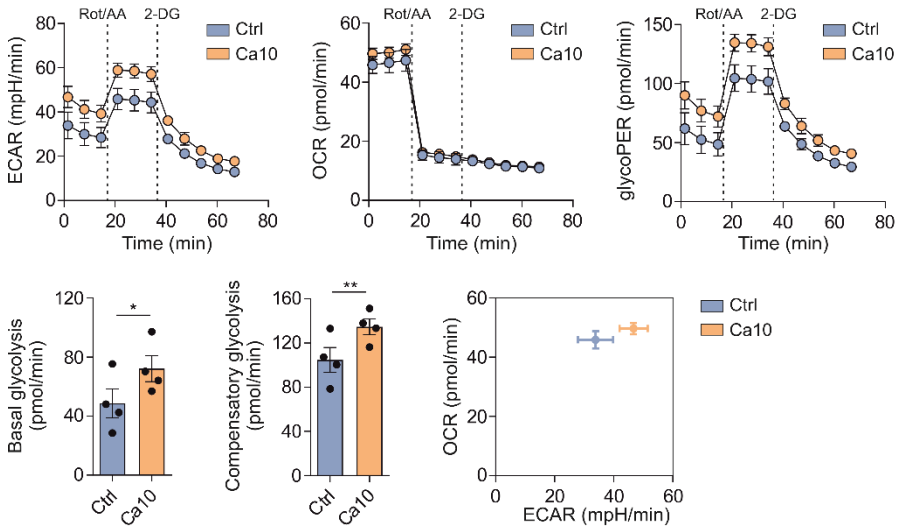

Figure 69. Ca10-activated hmoDCs exhibit a metabolic rewiring with an increased glycolysis. Kinetic study of extracellular acidification rate (ECAR), mitochondrial oxygen consumption rate (OCR), glycolytic proton efflux rate (glycoPER) and energy map in medium (ctrl) or Ca10-stimulated hmoDCs by sequential addition of rotenone/antimycin A (Rot/AA) and 2-Deoxy-D-glucose (2-DG). Quantification of basal glycolysis and compensatory glycolysis of hmoDCs are included (n = 4 of two independent experiments). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Supporting this result, we did not observe significant changes in basal respiration, maximal respiration, ATP production coupled to respiration or spare respiratory capacity between Ca10-activated DCs and unstimulated DCs (**Figure 70**), suggesting that mitochondrial OXPHOS was not altered by Ca10 stimulation.

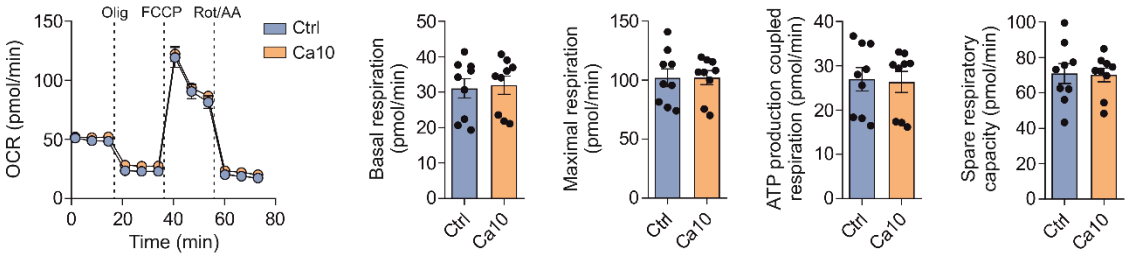

Figure 70. Analysis of OXPHOS in hmoDCs stimulated with Ca10. Kinetic study of mitochondrial oxygen consumption rate (OCR) in medium (ctrl)- or Ca10-stimulated human DCs by sequential addition of Oligomycin (Olig), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and rotenone/antimycin A (Rot/AA). Quantification of basal respiration, maximal respiration, ATP production coupled respiration and spare respiratory capacity of human DCs are included (n = 9 of three independent experiments). Values are mean ± SEM.

HmoDCs stimulated with Ca10 significantly increased the consumption of glucose from the culture medium and the metabolic rate compared to unstimulated cells (**Figure 71A**), supporting the previous data. Accordingly, Ca10-activated human DCs

showed a significantly higher Warburg effect and lactate production in cell culture supernatants than control cells (**Figure 71B and C**).

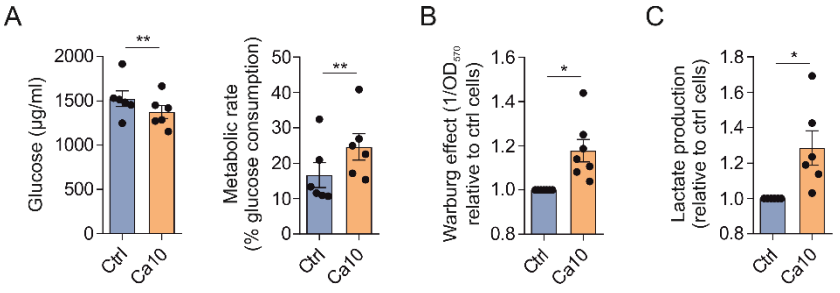

Figure 71. Ca10-activated hmoDCs display a higher glucose consumption, Warburg effect and lactate production. (A) Glucose consumption by human DCs treated with medium (ctrl) or Ca10 for 18 hours and calculated metabolic rate (percentage of glucose consumption) (n = 6). (B) Quantification of the induced Warburg effect relative to control cells (n = 7). (C) Increase in lactate production in cell-free supernatants from Ca10-stimulated human DCs relative to unstimulated cells (n = 6). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, and ***P* < 0.01.

Collectively, these data indicated that Ca10 enhances glycolysis without altering mitochondrial OXPHOS, indicating that Ca10 induces metabolic reprogramming in human DCs characterized by a shift to glycolysis with significant lactate production, which might also contribute to the reported tolerogenic features (**Figure 68**). It has been previously shown that lactate impairs anti-tumor immunity within the TEM by different mechanisms including direct suppression of DCs (111, 276-278). Tolerogenic DCs produce high levels of lactate which supress proliferation of effector T cells and induce Treg cells (117). Lactate accumulation within the TME also directly supports tumor-infiltrating functional Treg cells via metabolic adaptations, thus enhancing tumor survival (98).

Then, we wanted to analyse the contribution of mTOR and glycolysis to the tolerogenic properties exerted by the carbohydrate Ca10 in human DCs. The inhibition of mTOR pathway with rapamycin and glycolysis with 2-Deoxy-D-glucose (2-DG), a synthetic analogue of glucose, in Ca10-activated hmoDCs significantly suppressed the production of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β as well as IL-10, whereas a significant increase in the production of TNFα was observed in the presence of 2-DG (**Figure 72A**). Accordingly, inhibition of mTOR and glycolysis significantly reduced the expression of PD-L1 induced by Ca10 in hmoDCs (**Figure 72B**), reflecting that glycolytic metabolism and mTOR-mediated signalling pathways contribute to the tolerogenic properties imprinted by the carbohydrate Ca10 in human DCs.

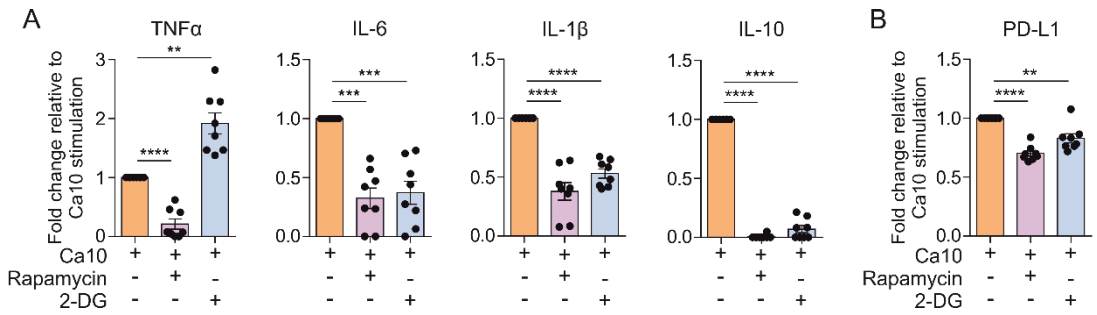

Figure 72. Contribution of mTOR pathway and glycolysis in cytokine production and PD-L1 expression induced by Ca10 in hmoDCs. (A) Cytokine production of hmoDCs stimulated with Ca10 in the presence of mTOR inhibitor (rapamycin) or glycolysis inhibitor (2-Deoxy-D-glucose, 2-DG) for 18 hours relative to Ca10 stimulation (n = 8). (B) Mean fluorescence intensity values for the surface marker PD-L1 on hmoDCs after stimulation with Ca10 in the presence of rapamycin or 2-DG for 18 hours relative to Ca10 stimulation (n = 8). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

3.5. To study the capacity of the tumoral carbohydrate Ca10 to generate Treg cells and the underlying molecular mechanisms involved in such effects

To determine whether Ca10 could indeed generate human tolerogenic DCs with the capacity of polarize functional Treg cells, we performed allogeneic coculture experiments with Ca10-activated hmoDCs and allogeneic naïve $CD4^+$ T cells. After 5 days of coculture, Treg cell generation was assessed by flow cytometry and T cell cytokine production was quantified by ELISA. As shown in **Figure 73**, Ca10-stimulated hmoDCs induced significantly higher percentage of $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells than unstimulated control hmoDCs.

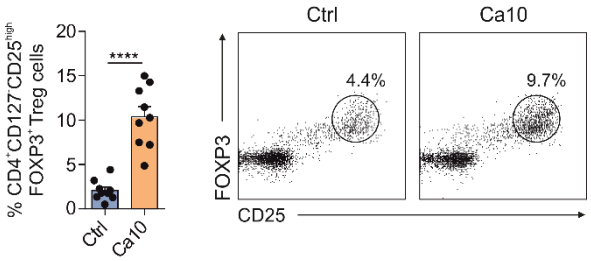

Figure 73. Ca10-activated hmoDCs generate $FOXP3^+$ Treg cells. Percentage of induced $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells gated on $CD4^+$ T cells by untreated (ctrl) or Ca10-treated hmoDCs after 5 days (n = 9). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. *****P* < 0.0001.

Then, we wanted to investigate whether antibody A10 was also able to impair the capacity of Ca10-activated hmoDCs to promote Treg cell generation. For that, the carbohydrate Ca10 was previously incubated with the antibody A10 or corresponding isotype control (in a ratio of 1:10 or 1:5, Ca10:A10) for 1 hour and after that, hmoDCs

were stimulated for 18 hours. Then we performed the same type of coculture experiments. Remarkably, in the presence of A10, the number of $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells induced by Ca10-activated hmoDCs was significantly decreased (**Figure 74**).

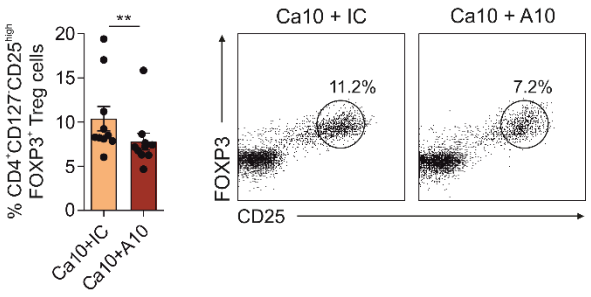

Figure 74. The antibody A10 impairs the induced Treg cells by Ca10-activated hmoDCs. Percentage of induced $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells gated on $CD4^+$ T cells by hmoDCs treated with Ca10 in the presence of the monoclonal antibody A10 or the corresponding isotype control (IC) after 5 days (pool of n = 10 of 1:10 and 1:5 ratios, Ca10:A10). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. ***P* < 0.01.

To assess the suppressive capacity of Treg cells generated by hmoDCs stimulated with Ca10, functional suppression assays were performed. The fraction of $CD4^+CD127^-CD25^+$ Treg cells generated by Ca10-activated hmoDCs was sorted by fluorescence-activated cell sorting (FACS) and cocultured with anti-CD3/CD28-stimulated autologous PBMC to analyse the proliferation of PBMC by CFSE-dilution experiments by flow cytometry. Remarkably, the induced Treg cells by Ca10-stimulated hmoDCs were functional as they were able to potently suppress, in contrast to the non-Treg cell fraction, the proliferation of autologous PBMC in a dose-dependent manner (**Figure 75A and B**).

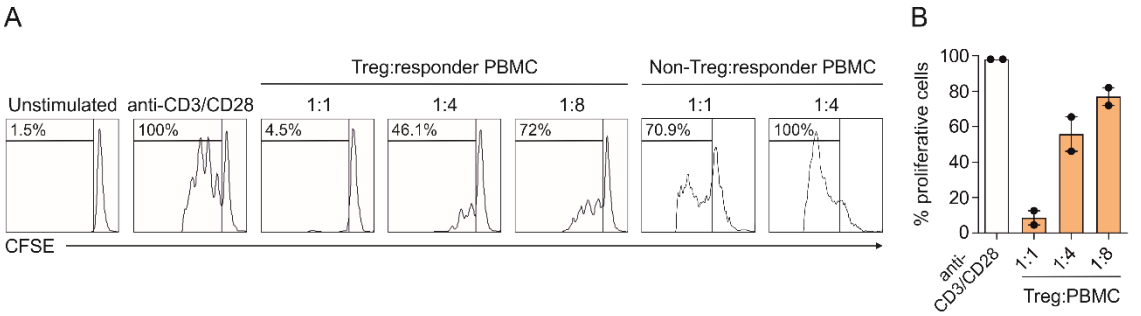

Figure 75. Ca10-activated hmoDCs generate functional suppressive Treg cells. (A) Suppression assays of purified Treg cells and non-Treg cells generated by Ca10-activated hmoDCs. (B) Percentage of proliferating $CD4^+$ cells after 5 days of stimulation in the presence of autologous purified Treg cells generated by Ca10-activated hmoDCs at different ratios (n = 2). Values are mean ± SEM.

Then, we wanted to confirm the Treg cell generation in total DCs from human peripheral blood of healthy donors. The enriched fraction of human blood total DCs containing both plasmacytoid (pDC) and myeloid (mDC) DCs (**Figure 76A**) stimulated with Ca10 also generated significantly higher numbers of $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells than unstimulated total DCs (**Figure 76B**), supporting the findings previously described in hmoDCs.

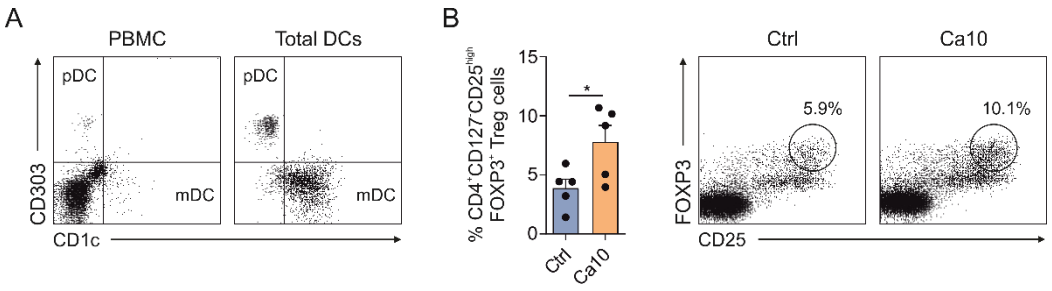

Figure 76. Ca10-activated total DCs generate $FOXP3^+$ Treg cells. (A) Representative dot plots of different DCs subsets in PBMCs and total DC fraction gated on $HLA\text{-}DR^+CD19^-$ cells. Total DC fraction containing both $HLA\text{-}DR^+CD1c^+$ myeloid (mDC) and $HLA\text{-}DR^+CD303^+$ plasmacytoid DCs (pDC) are shown. (B) Percentage of induced $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells gated on $CD4^+$ T cells by untreated (ctrl) or Ca10-treated total blood DCs after 5 days (n = 5). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. *$P < 0.05$.

To assess the relevance of the tolerogenic molecules PD-L1, IL-10 and IDO, as well as mTOR pathway and glycolysis induced by Ca10 in hmoDCs for the polarization of T cell responses, we performed coculture experiments. As shown in **Figure 77A**, blocking of PD-L1, IL-10, IDO, mTOR and glycolysis significantly impaired the generation of Treg cells by Ca10-stimulated hmoDCs. In addition, T cell primed by Ca10-activated hmoDCs in the presence of anti-PD-L1, anti-IL-10 or 1-MT, produced significantly lower IFNγ concentration (especially after PD-L1 inhibition), whereas the presence of rapamycin and 2-DG significantly decreased IFNγ production. This was accompanied by significantly lower levels of IL-5 and IL-10 in the presence of all assayed inhibitors, without differences for IL-10 production after blocking PD-L1 (**Figure 77B**).

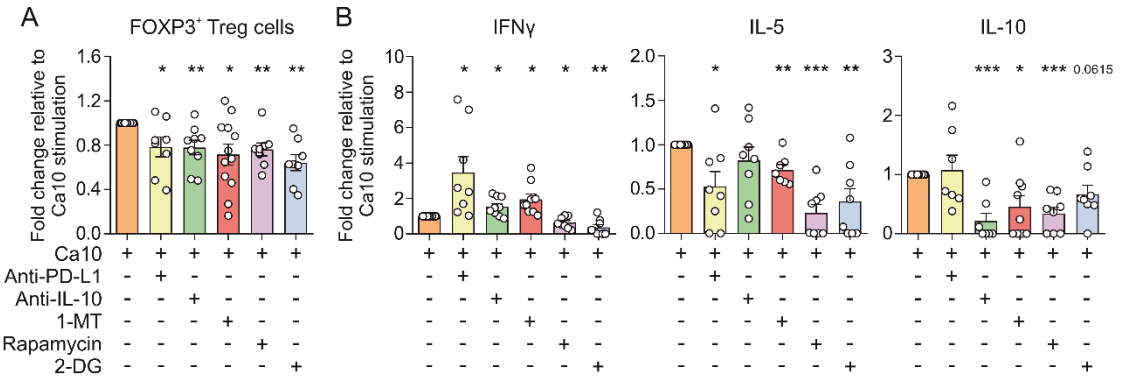

Figure 77. Ca10-activated hmoDCs induce Treg cells depending on PD-L1, IL-10, IDO, mTOR pathway and glycolysis. (A) Percentage of $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells induced by Ca10-treated hmoDCs in the presence of PD-L1, IL-10 blocking antibody, 1-MT (IDO inhibitor), rapamycin (mTOR inhibitor) or 2-DG (glycolysis inhibitor) after 5 days relative to Ca10-stimulation alone (n = 8-12). (B) Cytokines produced by allogeneic naïve $CD4^+$ T cells primed by Ca10-activated hmoDCs after 5 days under the indicated conditions relative to Ca10-stimulation alone (n = 7-9). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

In summary, the carbohydrate Ca10 endorses human DCs with the capacity to generate highly suppressive Treg cells by mechanisms partially depending on PD-L1, IL-10 and IDO, as well as mTOR pathway and glycolysis. Blocking of PD-L1 on Ca10-stimulated DCs significantly increases the levels of IFNγ in T cell coculture experiments, indicating that Ca10-induced PD-L1 on human DCs is essential to favour the generation of suppressive Treg cells vs IFN-γ-producing T cells, which are also key effector cells to mount proper anti-cancer immune responses (218).

3.6. To assess the specific contribution of the carbohydrate moiety of Ca10 and the epitope A10 to condition the capacity of DCs to polarize T cell responses, with special focus on the generation of Treg cells

The carbohydrate Ca10 was initially described as a highly glycosylated ET cell surface macromolecule defined by the binding of monoclonal A10 (244, 245). In some human cancer, the antibody A10 also reacts with glycan moieties located in cell surface mucins (250). However, the structural features of the carbohydrate moiety contained in Ca10 and its potential functional implications in cancer remains largely unknown.

The group of Dr. Francisco Javier Cañada from Centro de Investigaciones Biológicas Margarita Salas (CIB, Madrid, Spain), has uncovered the structure of Ca10 by using nuclear magnetic resonance (NMR). Oxidation treatment of Ca10 with $NaIO_4$ completely eliminated the reactivity of the A10 and resulted in significant degradation and molecular size reduction of the carbohydrate component as observed by NMR diffusion ordered spectroscopy. Since the NMR profiles of Ca10 saccharides are

consistent with glycosaminoglycans (GAGs), enzymes degrading glycans were used. In particular, treatment of Ca10 with Heparinase II/III completely abolished A10 reactivity and also hydrolyzed the carbohydrate component into low molecular weight fragments as determined by diffusion NMR. The NMR profile of the major fraction of the resulting disaccharide fragments upon digestion matches the profiles of ΔUA-GlcNH2 and ΔUA-GlcHNHAc related to heparan sulfate-forming disaccharides, indicating that the main carbohydrate moiety of Ca10 is a glycosaminoglycan of heparan type (unpublished data).

Heparan sulfates are covalently attached to a core protein forming proteoglycans (279). The biosynthesis of heparan sulfate proteoglycans starts with the assembly of a tetrasaccharide linkage region at GAG attachment sites on the core protein. After that, the heparan sulfate chain is polymerized by the addition of alternating residues of glucuronic acid (GlcA) and N-acetylglycosamine (GlcNAc). Finally, the elongated chain suffers several modifications including N-deacetylation/N-sulfation, O-sulfation or iduronic acid formation, which results in the synthesis of heparan sulfate chains with high structural variability (279, 280). Heparan sulfate proteoglycans display essential functions in the maintenance of extracellular matrix, cell adhesion, cellular growth, proliferation, migration, inflammation or pathogen invasion (279, 281), while in cancer these molecules mediate tumor progression, angiogenesis and metastasis (282, 283). The aberrant expression of the enzymes involved in heparan sulfate synthesis results in aberrant heparan sulfate-related structures, directly involved in malignant transformation in different types of cancer, which can potentially serve as target for cancer therapies (280).

We wanted to analyse the contribution of the heparan sulfate-related proteoglycan in the observed tolerogenic properties imprinted by Ca10 in hmoDCs. For that, hmoDCs were stimulated with Ca10, Ca10 oxidized with $NaIO_4$ (Ca10 OX) or Ca10 treated with Heparinase II/III (HPSE treat.) to assess IL-10 production and PD-L1 expression. In addition, hmoDCs were preincubated with heparin and then, stimulated with Ca10. Heparin has similarities in structure with the molecule of heparan sulfate and therefore, it can be used as an heparan sulfate mimetic to perform a competition assay for the heparan sulfate binding sites (284). Oxidation of Ca10, Heparinase treatment or the presence of heparin abolished Ca10-mediated IL-10 production and significantly impaired PD-L1 expression in hmoDCs (**Figure 78A and B**).

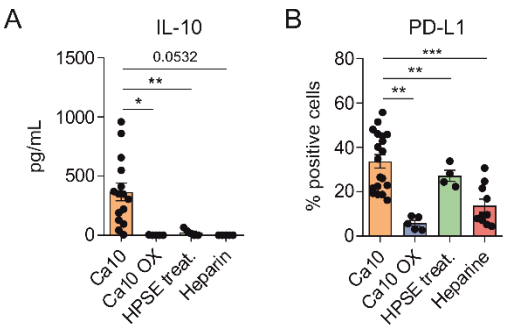

Figure 78. Effect of oxidation of Ca10, Heparinase treatment and the presence of heparin in IL-10 production and PD-L1 expression in Ca10-activated hmoDCs. (A) IL-10 production and (B) percentage of PD-L1^{+} hmoDCs stimulated with Ca10, Ca10 oxidized with $NaIO_4$ (Ca10 OX), Ca10 treated with Heparinase (HPSE treat.) or Ca10 in the presence of heparin for 18 hours (n = 4-10). Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. *P < 0.05, and **P < 0.01.

To assess how the different treatments of Ca10 or the presence of heparin affected the capacity of hmoDCs to polarize T cells, the induction of Treg cells was analysed. Similarly, the generation of CD4^{+}CD127^{-}CD25^{high}FOXP3^{+} Treg cells by Ca10-stimulated hmoDCs was significantly reduced when human DCs were stimulated with Ca10 previously subjected to oxidation with $NaIO_4$, treated with Heparinase or in the presence of heparin (**Figure 79**).

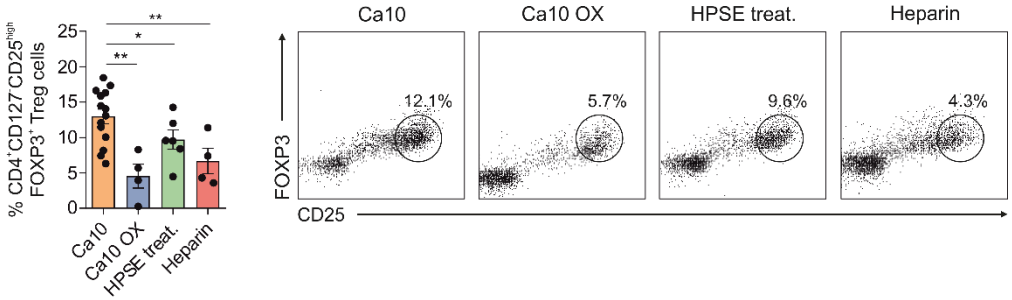

Figure 79. Oxidation of Ca10, Heparinase treatment and the presence of heparin impair Treg cell generation by Ca10-activated hmoDCs. Percentage of CD4^{+}CD127^{-}CD25^{high}FOXP3^{+} Treg cells gated on CD4^{+} T cells generated after 5 days of priming by hmoDCs stimulated with Ca10, Ca10 oxidized (Ca10 OX), Ca10 treated with Heparinase (HPSE treat.) or Ca10 in the presence of heparin (n = 4-6). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. *P < 0.05, and **P < 0.01.

Collectively, these data demonstrated that the heparan sulfate glycosaminoglycan moieties contained in the carbohydrate structure of Ca10 imprints tolerogenic features in human DCs. Although different studies previously showed that DCs within the TME may become tolerogenic and favours tumor progression through different mechanisms (239, 285, 286), in this study we demonstrate, for the first time, that a heparan sulfate-related glycosaminoglycan structure from a tumoral carbohydrate promotes human tolerogenic DCs able to generate highly suppressive Treg cells.

Objective 4. Study of the capacity of the tumoral carbohydrate Ca10 to reprogram monocyte differentiation into tolerogenic DCs

During tumor progression, monocytes are recruited to tumor site for differentiation into macrophage or DCs (74, 75, 216, 287). Depending on the signals encountered in the TME, monocytes might well differentiate into tolerogenic DCs (74, 82, 288). We previously showed the generation of tolerogenic human DCs by Ca10 from already differentiated immature DCs; however, the effect of the carbohydrate Ca10 in the differentiation process from monocytes to DCs still remains unknown. In this chapter, we investigated the phenotype and function of hmoDCs generated from monocytes in the presence of Ca10.

4.1. To study whether the tumoral carbohydrate Ca10 could reprogram human monocytes differentiation into tolerogenic DCs

To study how the carbohydrate Ca10 could influence this differentiation process, purified blood monocytes from healthy donors were differentiated into hmoDCs in the presence or absence of Ca10, which was added at days 0 and 4 of the differentiation, as previously described in Materials and Methods section. After 6 days, activation and functional markers expression was analysed by flow cytometry and tolerogenic gene expression were assessed by qPCR.

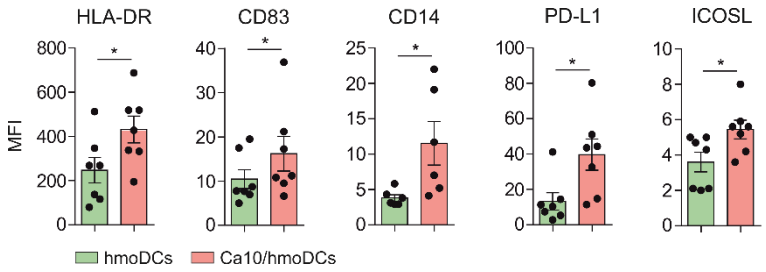

Figure 80. Ca10/hmoDCs upregulate the expression of HLA-DR, CD83, CD14, PD-L1 and ICOSL. Mean fluorescence intensity (MFI) values for the surface markers HLA-DR, CD83, CD14, PD-L1 and ICOSL in conventional hmoDCs or Ca10/hmoDCs (n = 6-7). Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. *$P < 0.05$.

HmoDCs generated in the presence of Ca10 (Ca10/hmoDCs) expressed significantly higher levels of HLA-DR, CD83, CD14 and the tolerogenic markers PD-L1 and ICOSL than conventional hmoDCs generated in absence of Ca10 (**Figure 80**). Ca10/hmoDCs expressed significantly higher levels of MHC class II (HLA-DR) and costimulatory molecule CD83 than conventional hmoDCs (28, 252). The classical monocyte marker CD14 is downregulated in response to IL-4 and GM-CSF during the differentiation process into DCs (289). However, CD14 expression in Ca10/hmoDCs was

maintained upon their differentiation from monocytes, which has been described for tolerogenic DCs induced in the presence of IL-10, dexamethasone or vitamin D3 (290, 291). It has been described in prostate cancer that TME-derived factors induce the differentiation from monocytes into DCs upregulating CD14 as well as PD-L1 expression (82). PD-L1 and ICOSL are immune checkpoints that limit T cell activation, thus inducing tolerance (37, 40, 84).

As shown in **Figure 81**, Ca10/hmoDCs expressed higher *PDL1*, *IDO*, *SOCS1* and *SOCS3* tolerogenic genes at mRNA level compared to hmoDCs, while *RALDH1* and *RALDH2* gene expression was decreased. All these molecules are involved in the generation of Treg cells, as previously described (99, 101).

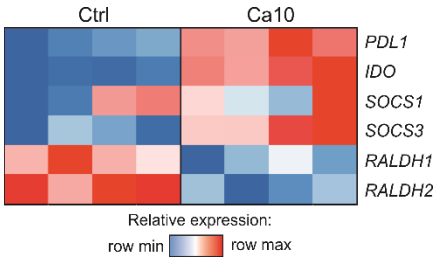

Figure 81. Ca10/hmoDCs express tolerogenic molecules at mRNA level. Heatmap representation for gene expression on freshly generated conventional hmoDCs or Ca10/hmoDCs (n = 4).

To analyse the capacity of Ca10/hmoDCs to produce cytokines in response to stimulation, human DCs were stimulated with LPS for 18 hours. Ca10/hmoDCs produced a significantly lower concentration of pro-inflammatory cytokines TNFα, IL-6 and IL-1β as well as the anti-inflammatory IL-10 (**Figure 82A**). Despite the lower production of IL-10 by Ca10/hmoDCs after LPS stimulation, the TNFα/IL-10, IL-6/IL-10 and IL-1β/IL-10 ratios were significantly lower in Ca10/hmoDCs compared to hmoDCs (**Figure 82B**). These results suggested that during the differentiation process, Ca10 might imprint an anti-inflammatory phenotype in hmoDCs.

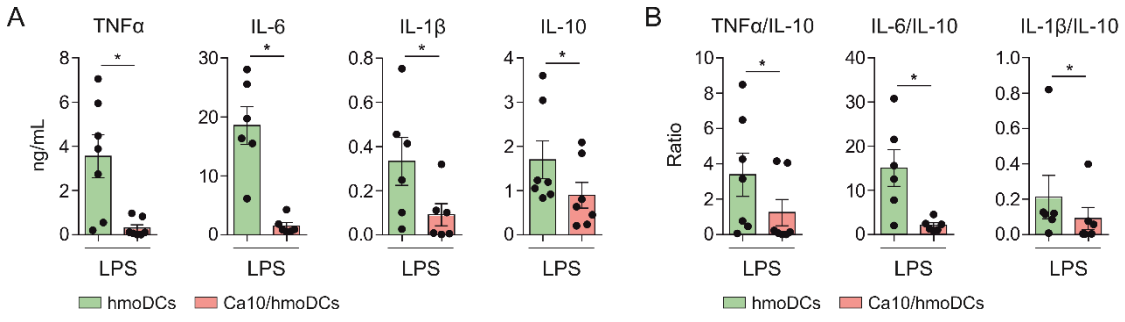

Figure 82. Ca10/hmoDCs display a reduced capacity to produce cytokines after stimulation. (A) Cytokine production after stimulation of hmoDCs or Ca10/hmoDCs with LPS for 18 hours in cell-free supernatants (n = 6-7). (B) TNFα/IL-10, IL-6/IL-10 and IL-1β/IL-10 ratios produced by hmoDCs or Ca10/hmoDCs after 18 hours of stimulation with LPS (n = 6-7). Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. *$P < 0.05$.

Then, we wanted to analyse the metabolic state of Ca10/hmoDCs compared to conventional hmoDCs. We performed functional metabolic experiments using a Seahorse bioanalyzer to monitor real-time mitochondrial OCR after 24 hours of resting in medium. Ca10/hmoDCs showed a significant decrease basal and maximal respiration, ATP production coupled respiration and spare respiratory capacity compared to hmoDCs (**Figure 83**), suggesting that Ca10/hmoDCs displayed a lower mitochondrial OXPHOS activity than conventional hmoDCs.

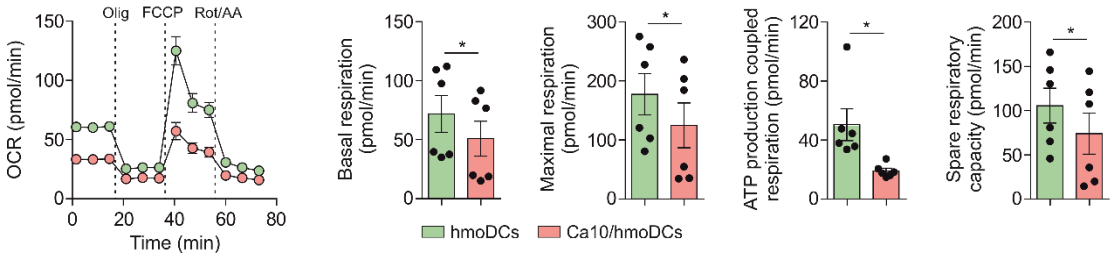

Figure 83. Ca10/hmoDCs display a reduced mitochondrial OXPHOS. Kinetic study of mitochondrial oxygen consumption rate (OCR) in conventional hmoDCs or Ca10/hmoDCs by sequential addition of Oligomycin (Olig), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) and rotenone/antimycin A (Rot/AA). Quantification of basal respiration, maximal respiration, ATP production coupled respiration and spare respiratory capacity of human DCs are included (n = 6 of two independent experiments). Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. *$P < 0.05$.

4.2. To study the capacity of hmoDCs differentiated in the presence of Ca10 to polarize Treg cells

Several studies have described that tolerogenic DCs generated in TME from monocytes mediate Treg cell generation (292, 293). Then, we assessed the capacity of Ca10/hmoDCs to polarize T cell responses. For that, coculture experiments with conventional hmoDCs or Ca10/hmoDCs and allogeneic naïve $CD4^+$ T cells were performed. After 5 days of coculture, cytokine production by T cells were quantified. Interestingly, Ca10/hmoDCs generated T cells producing significantly lower concentration of IFNγ than T cells primed by conventional hmoDCs, without differences in IL-5 or IL-10 production (**Figure 84A**). The IL-10/IFNγ ratio was significantly higher when T cells were generated by Ca10/hmoDCs than conventional hmoDCs (**Figure 84B**). No differences were observed in IL-10/IL-5 ratio. These data suggested that the decrease in IFNγ production by T cells primed with Ca10/hmoDCs might also contribute to impair the anti-tumor response of macrophages, NK cells and CTLs, as well as to downregulate the expression of MHC class I and II by tumor cells and the tumor-derived antigen presentation pathways (211, 218).

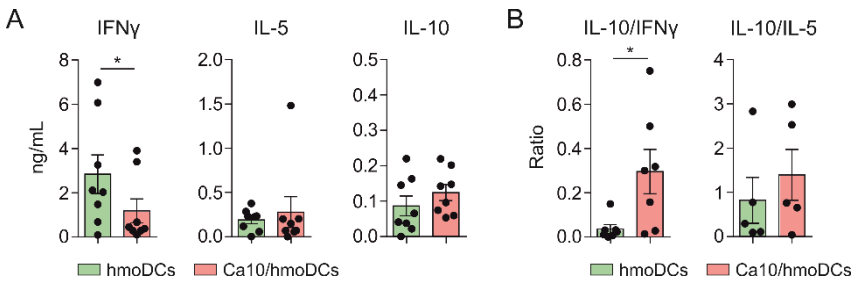

Figure 84. Ca10/hmoDCs generate T cells producing lower levels of IFNγ than hmoDCs. (A) Cytokines produced by allogeneic naïve $CD4^+$ T cells primed by hmoDCs or Ca10/hmoDCs after 5 days quantified in cell-free supernatants (n = 8). (B) IL-10/IFNγ and IL-10/IL-5 ratios produced by T cells primed by hmoDCs or Ca10/hmoDCs (n = 5-7). Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. *$P < 0.05$.

To assess the generation of Treg cells, the same type of coculture experiments were performed to analyse the intranuclear expression of FOXP3. Ca10/hmoDCs induced a significant higher percentage of $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells than conventional hmoDCs (**Figure 85**). These results suggested that Ca10 might promote the differentiation of monocyte recruited into the TME into tolerogenic DCs with an anti-inflammatory phenotype and the capacity to generate Treg cells while decreasing IFNγ-producing T cell responses, which could represent a mechanism of tumor escape.

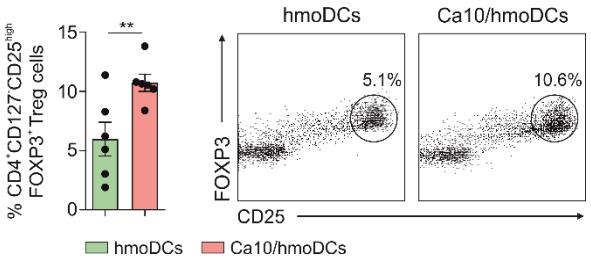

Figure 85. Ca10/hmoDCs generate $FOXP3^+$ Treg cells. Percentage of induced $CD4^+CD127^-CD25^{high}FOXP3^+$ Treg cells gated on $CD4^+$ T cells by conventional hmoDCs or Ca10/hmoDCs after 5 days (n = 6). Representative dot plots of intranuclear FOXP3 staining are shown. Values are mean ± SEM. Statistical significance was determined using Paired Student *t* test. **$P < 0.01$.

In summary, we provide novel insight into the molecular mechanism by which the carbohydrate Ca10 derived from ET cells bears heparan sulfate-related structure that promotes tolerogenic human DCs able to generate highly suppressive Treg cells by mechanisms partially depending on tolerogenic molecule induction and metabolic reprogramming. In addition, our *in vivo* data demonstrate positive correlations between concentration of circulating Ca10, tumor size and the induction of Treg cells in mice bearing solid ET, being the carbohydrate Ca10 involved in this Treg cell generation. Additionally, Ca10 reprograms the differentiation of monocytes into DCs with tolerogenic features. Our finding might well contribute to elucidate the potential implications of

carbohydrates bearing heparan sulfate-related structures in cancer and might well pave the way for the identification of potential novel tumor biomarkers and for the improvement or development of alternative therapeutic strategies in cancer.

Global Discussion

DCs play a key role in the orchestration of proper immune responses by connecting innate and adaptive immunity (21, 22). Under normal conditions, DCs develop potent immune responses to clear potentially dangerous pathogens and to induce immune tolerance to self- and non-self-innocuous antigens (21, 25, 26). Alterations of this vital function might lead to different immune-mediated diseases such allergy, autoimmunity, recurrent infections or cancer. The capacity of DCs to initiate and sustain proper immune responses can be modulated by exogenous signals. Therefore, DCs are regarded as promising therapeutic targets for different immune-mediated diseases such as recurrent infections or cancer.

Bacterial and fungal infectious diseases represent major healthcare problems of increasing prevalence with high socio-economic burden. Among them, recurrent GUTIs including RUTIs and RVVCs affect to millions of people worldwide, significantly impairing patient's quality of life (132-134). Nowadays, the mainstay of treatments are antibiotics and antifungals, respectively, but their widespread use is associated with microbial resistances and microbiota deleterious effects (133, 135-138). The prolonged antibiotic therapy for RUTIs has been directly associated with vagina microbiota alterations, thus increasing the risk to develop VVCs (137, 160, 161). Sublingual microbial vaccines have been postulated as alternative therapeutic strategies to prevent recurrent infections (152, 183, 187, 189). The whole heat-inactivated polyvalent bacterial preparation MV140 has demonstrated clinical efficacy for the treatment of RUTIs (152, 192-199). It has been previously described that MV140 acts on human DCs and promotes *in vitro* and *in vivo* effective Th1, Th17 and IL-10-producing T cell responses (78). Regarding RVVCs, different *Candida* vaccines have been assessed, but none based on the inactivated pathogen (169, 200). The whole heat-inactivated *C. albicans* V132 preparation has been developed as therapeutic strategy, but the mode of action of V132 has not been fully explored yet.

Given the close relationship between RUTIs and RVVCs the combined treatment with MV140 and V132 might represent a suitable approach for the prevention of recurrent episodes. Here, we have demonstrated that the combination of MV140 and V132 in a single preparation endorses human DCs with the capacity to induce IFNγ- and IL-17A-producing T cells for pathogen clearance, as well as Treg cells to suppress excessive immune responses, favoring tissue repair and homeostasis (90, 253, 255, 257, 258). Different studies have reported that the function of immune cells is tightly related to metabolic and epigenetic rewiring (17, 125). We reported that MV140/V132 shifts the glucose metabolism towards an increased glycolysis and induces the histone methylation in the genes of cytokines TNFα, IL-23 and IL-10 in DCs (**Figure 86**). The

potential *in vivo* relevance of MV140/V132 to promote adaptive immune responses was assessed in a mice model. We have reported that MV140/V132 sublingual immunization of mice induces the production of specific antibodies and the proliferation of splenic $CD4^+$ T cells against the components contained in the vaccine. In addition, an enhanced proliferation rate was observed in splenic T cells against non-related antigens, indicating that MV140/V132 might confer protection against heterologous infections (**Figure 86**). This result together with the metabolic and epigenetic changes suggest that MV140/V132 could promote trained immunity (10, 263).

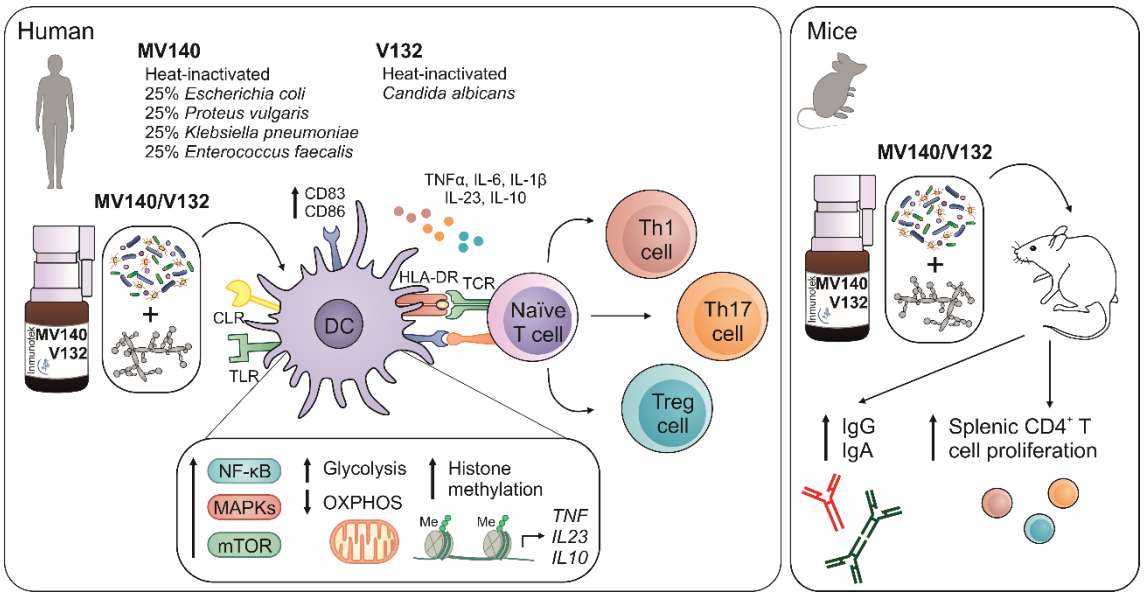

Figure 86. MV140/V132 immunomodulates human DCs and enhances *in vivo* adaptive immune responses. Simultaneous stimulation of human DCs with the combination of MV140 and V132 promotes the generation of IFNγ- and IL-17A-producting T cells as well as Treg cells. Sublingual immunization of mice with MV140/V132 enhances proliferation of splenic $CD4^+$ T cells and specific IgG and IgA antibody production.

Vaccination promotes adaptive immune memory, but there is a strong evidence that some vaccines are also able to induce memory in innate immune cells by a trained immunity mechanism, which confer broader protection against heterologous pathogens (172, 173, 175). Several vaccine formulations containing live attenuated or inactivated pathogens have been proposed as TIbV (11, 18, 19, 177-182). Here, we demonstrated that V132 induces trained immunity in human monocytes enhancing TNFα and IL-6 production against a second stimulation with MV140. V132-trained monocytes reprogram their metabolism towards a higher glycolysis and mitochondrial oxidative phosphorylation than untrained cells. In addition, V132 promotes epigenetic rewiring at histone marks that drives the transcription of the pro-inflammatory genes *TNFA* and *IL6* (**Figure 87**). We have observed that V132 sublingual immunization of mice induces

trained of peritoneal cells and splenocytes, enhancing the immune responses triggered by *in vitro* MV140. In contrast, MV140 and MV140/V132 did not induced trained immunity, as expected for MV140 formulation containing 75% of Gram-negative bacteria (123, 260, 264). However, the combination of MV140 together with V132 formulation allows to incorporate bacterial antigens to induce adaptive immune responses against all stimuli. In fact, here we have also shown that previous V132 immunization potentiates Th17 responses in MV140-treated mice (**Figure 87**). Although it has been described in the last years that *C. albicans* and its cell wall component β-glucan are trained immunity inducers (4, 120), here we demonstrated, for the first time, that V132 might be used for the prevention of RVVCs as well as for GUTIs in which RUTIs coexist (135).

In summary, these studies shed light into the underlying mechanisms by which the bacterial MV140 and fungal V132 sublingual vaccines immunomodulate the function of human DCs and monocytes. Our findings might well contribute to develop novel therapeutic alternative for GUTIs as well as other immune-related diseases.

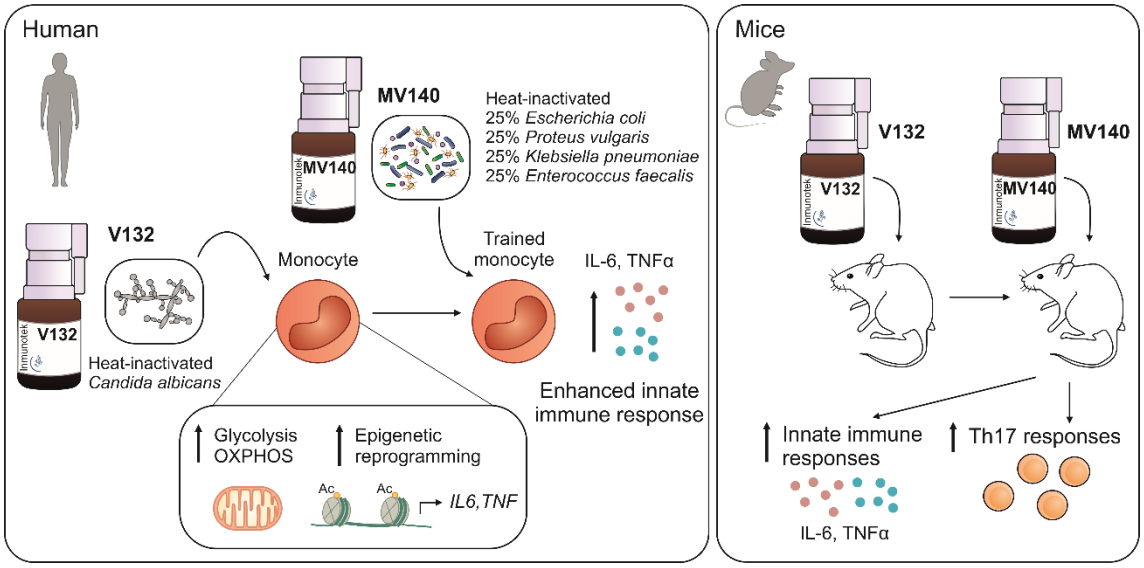

Figure 87. Trained immunity induction by V132 enhances innate immune responses and potentiates Th17 responses upon treatment with MV140. V132 induces trained immunity in human monocytes, which undergo metabolic and epigenetic rewiring, thus enhancing the responses upon a subsequent stimulation with MV140. Sublingual immunization of mice with V132 potentiates innate immune responses and increases Th17 responses upon stimulation with MV140.

Cancer represents, depending on the country, the first or second main cause of death worldwide, and the prevalence is dramatically increasing over the last decades (204). Cancer cells promote a tolerant TME favoring tumor progression and dissemination (226, 294). Functional Treg cells are abnormally expanded in cancer and greatly increased within the tumor tissues, playing a key role in tumor development and

progression as well as a major barrier to anti-cancer immunity (98, 225). The ability of mature DCs infiltrating tumors to recognize tumor antigens and promote suppressive Treg cells is highly influenced by exogenous signals encountered in the TME (21). During the process of malignant transformation, cancer cells display changes in the cell surface glycosylation pattern, thus resulting in aberrant or incomplete glycosylation structures (231, 232). These glycoconjugates generate new tumor-associated carbohydrates having a strong influence on tumor progression and inhibiting anti-tumor responses (234, 236-239). The aberrant tumoral carbohydrate Ca10 overexpressed in ET cells and shed from tumor cells might be related with tumor progression (244, 245).

In this study, we have observed *in vivo* positive correlation between circulating Ca10 levels in serum, tumor size and the induction of splenic Treg cells in a solid-ET bearing mice model, indicating that the release of Ca10 and Treg generation could represent mechanisms of tumor escape (**Figure 88**) (98, 225, 234, 245). In addition, the *in vivo* administration of Ca10 significantly increases the number of splenic Treg cells in mice, confirming the *in vivo* tolerogenic effect of this carbohydrate. Here we have demonstrated, for the first time, that the carbohydrate Ca10 induces tolerogenic human DCs with the capacity to generate highly suppressive Treg cells. We have observed that the DCs stimulated with Ca10 promote PD-L1 expression, IL-10 production and the expression of tolerogenic molecules *IDO, SOCS1* and *SOCS3*, which are molecules involved in the polarization of Treg cells (73, 86, 99, 275). In addition, Ca10 enhances glycolysis and lactate production without altering mitochondrial oxidative phosphorylation in DCs, which might also contribute to the reported tolerogenic features. It has been previously described that lactate accumulation in TME produced by tolerogenic DCs, among other cell types, impairs anti-tumor responses while inducing Treg cells (111, 117, 276-278). Mechanistically, Ca10 might trigger Syk-couple CLRs in a calcium-dependent manner, which activate MAPKs and NF-κB signalling pathways and, under certain circumstances, might promote tolerogenic DCs (38, 49, 51, 53). DCs stimulated with Ca10 polarize Treg cells by mechanisms partially depending on PD-L1, IL-10, IDO, mTOR pathway and glycolysis. Additionally, PD-L1 expression on DCs is involved not only in Treg cell generation, but also in decreasing the frequency IFNγ-producing T cells, which are also key effector cells to mount anti-cancer responses (**Figure 88**) (98, 100, 104, 211, 218). Supporting all these findings, the monoclonal antibody A10 impairs the generation of Treg cells induced by Ca10-activated DCs. Remarkably, our blocking *in vitro* experiments demonstrated that the heparan sulfate-related structure of the carbohydrate Ca10 is the structural moiety involved in the generation of tolerogenic DCs with the capacity to polarize functional Treg cells.

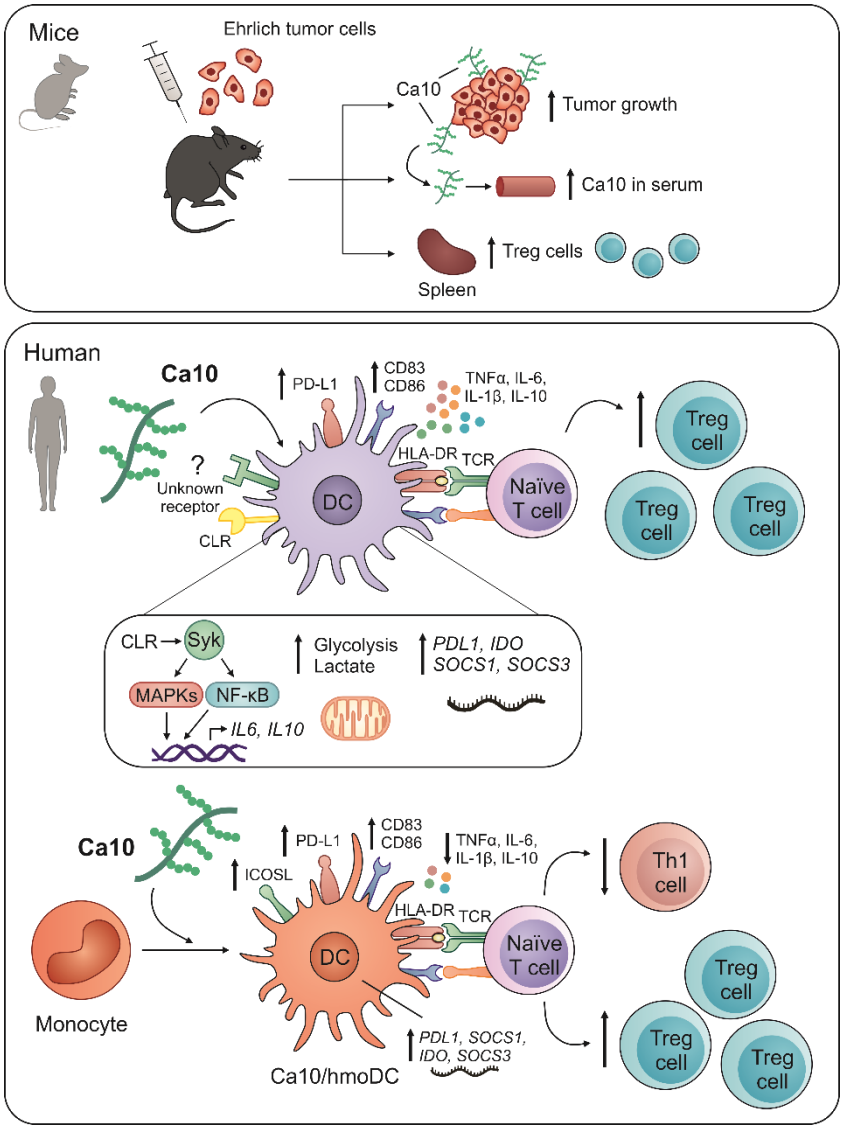

Figure 88. The tumoral carbohydrate Ca10 immunomodulates human DCs, thus generating Treg cells. In mice bearing solid ET, serum levels of Ca10 are positively correlated with tumor size and splenic Treg cells. The tumoral carbohydrate Ca10 promotes tolerogenic human DCs with the capacity to polarize highly suppressive functional Treg cells. Ca10 reprograms the differentiation of human monocytes into DCs with tolerogenic features.

During tumor progression, monocytes are recruited to tumor sites and the signals provided by TME influence their capacity to differentiate into tolerogenic DCs (74, 82, 288). It has been reported that tolerogenic DCs promotes the Treg cell generation while decreasing effector T cells within the tumor (292, 293). Here, we showed that Ca10/hmoDCs display a tolerogenic phenotype with increased *PDL1*, *ICOSL*, *IDO*, *SOCS1* and *SOCS3* expression and a reduced capacity to produce cytokines upon LPS stimulation. In addition, Ca10/hmoDCs promote a higher frequency of Treg cells and lower IFNγ-producing T cells than conventional hmoDCs, thus contributing to tumor progression (**Figure 88**) (98, 100, 104, 211, 218).

Collectively, our findings enhance the knowledge on how TEM-derived carbohydrate structures imprint tolerogenic DCs and promote suppressive Treg cells, which might well contribute to open new avenues of research with important clinical implications in cancer.

In summary, we uncover the molecular mechanisms by which human DCs exhibit different phenotypes and functions depending on the encountered stimuli. Bacterial and fungal sublingual vaccines exert beneficial effects on human DCs in the context of therapeutic strategies for recurrent infections. On the other hand, carbohydrate structures in TME promote tolerogenic DCs, thus inducing suppressive Treg cells and favoring the tumor progression. Overall, in this Doctoral Thesis we provide novel molecular insights into the regulation of human DC's function in the context of different immune-mediated diseases. Our findings might well contribute to develop new interventions for recurrent GUTIs as well as to open new avenues of research with important clinical implications in cancer.

CONCLUSIONS

The main conclusions of this Doctoral Thesis are:

Block A

1. The simultaneous stimulation with MV140/V132 induces the maturation of hmoDCs and a higher production of pro- and anti-inflammatory cytokines than the individual components separately. In addition, MV140/V132 imprints hmoDCs with the capacity to polarize potent IFNγ- and IL-17A-producting T cell as well as Treg cell responses.
2. MV140/V132 activates NF-κB, MAPKs and mTOR signalling pathways in hmoDCs. MV140/V132 induces a shift in glucose metabolism towards glycolysis and lactate production, as well as histone methylation in pro- and anti-inflammatory cytokine-related genes in hmoDCs.
3. Sublingual immunization of mice with MV140/V132 increases the proliferation of splenic $CD4^+$ T cells upon *in vitro* stimulation with related and non-related antigens. In addition, MV140/V132 immunization induces IgG and IgA antibodies against the specific components of the formulation in mice.
4. V132, but not MV140 or MV140/V132 treatment, induces the training of human monocytes and PBMC increasing pro-inflammatory cytokine production upon LPS stimulation. In addition, V132-trained monocytes display enhanced responses after MV140 secondary stimulation.
5. Training of monocytes with V132 is mediated by a shift in the glucose metabolism towards an enhanced glycolysis, lactate production and OXPHOS. Training of monocytes with V132 induces an epigenetic reprograming at the level of histone marks H3K27ac and H3K9me3 in the promoter regions of pro-inflammatory genes.
6. Peritoneal cells and splenocytes from mice intraperitoneally and sublingually immunized with V132 display enhanced innate immune responses upon *in vitro* stimulation with MV140. In addition, splenocytes from mice sublingually immunized with V132 and subsequent MV140 administration significantly increase Th17 responses upon *in vitro* MV140 stimulation.

Collectively, we reported that simultaneous stimulation of human DCs with the combination of MV140 and V132 promotes the generation of IFNγ- and IL-17A-producting T cells as well as Treg cells. V132 induces trained immunity in human monocytes, which undergo metabolic and epigenetic rewiring, thus enhancing the responses upon a subsequent stimulation with MV140.

Block B

1. Serum levels of Ca10 positively correlate with tumor size and with splenic Treg cell frequency in ET-bearing mice. In addition, mice administered with the tumoral carbohydrate Ca10 from ET display increased percentages of splenic Treg cells.
2. The tumoral carbohydrate Ca10 endorses tolerogenic features in hmoDCs characterized by the production of anti-inflammatory cytokine IL-10 and high expression levels of tolerogenic molecules.
3. The tumoral carbohydrate Ca10 activates MAPKs and NF-κB signalling pathways and induces a shift in glucose metabolism towards glycolysis and lactate production in hmoDCs. In addition, mTOR pathway and glycolysis are involved in the tolerogenic features imprinted by Ca10 in hmoDCs.
4. The tumoral carbohydrate Ca10 imprints hmoDCs with the capacity to generate functional Treg cells by mechanisms partially depending on PD-L1, IL-10, IDO, mTOR and glycolysis.
5. The heparan sulfate-related structure of Ca10 drives the induction of tolerogenic hmoDCs and Treg cell generation.
6. Monocytes differentiated into DCs in the presence of the tumoral carbohydrate Ca10 generate tolerogenic hmoDCs with the ability to promote Treg cells.

Collectively, we demonstrated that the tumoral carbohydrate Ca10 is an heparan sulfate-related proteoglycan that promotes tolerogenic human DCs with the capacity to polarize highly suppressive functional Treg cells. In mice bearing solid ET, serum levels of Ca10 are positively correlated with tumor size and splenic Treg cells. Ca10 reprograms the differentiation of human monocytes into DCs with tolerogenic features.

BIBLIOGRAPHY

1. Paludan SR, Pradeu T, Masters SL, and Mogensen TH. Constitutive immune mechanisms: mediators of host defence and immune regulation. *Nat Rev Immunol.* 2021;21(3):137-50.
2. Bonilla FA, and Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol.* 2010;125(2 Suppl 2):S33-40.
3. Iwasaki A, and Medzhitov R. Control of adaptive immunity by the innate immune system. *Nat Immunol.* 2015;16(4):343-53.
4. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, Jacobs L, Jansen T, Kullberg BJ, Wijmenga C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe.* 2012;12(2):223-32.
5. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de Jong D, Stunnenberg HG, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A.* 2012;109(43):17537-42.
6. Netea MG, Quintin J, and van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe.* 2011;9(5):355-61.
7. Divangahi M, Aaby P, Khader SA, Barreiro LB, Bekkering S, Chavakis T, van Crevel R, Curtis N, DiNardo AR, Dominguez-Andres J, et al. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. *Nat Immunol.* 2021;22(1):2-6.
8. Bekkering S, Dominguez-Andres J, Joosten LAB, Riksen NP, and Netea MG. Trained Immunity: Reprogramming Innate Immunity in Health and Disease. *Annu Rev Immunol.* 2021;39(667-93.
9. Dominguez-Andres J, Dos Santos JC, Bekkering S, Mulder WJM, van der Meer JWM, Riksen NP, Joosten LAB, and Netea MG. Trained immunity: adaptation within innate immune mechanisms. *Physiol Rev.* 2023;103(1):313-46.
10. Netea MG, Dominguez-Andres J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, Joosten LAB, van der Meer JWM, Mhlanga MM, Mulder WJM, et al. Defining trained immunity and its role in health and disease. *Nat Rev Immunol.* 2020;20(6):375-88.
11. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LA, Jacobs C, van Loenhout J, Xavier RJ, Aaby P, van der Meer JW, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J Innate Immun.* 2014;6(2):152-8.

12. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, van der Meer JW, van Crevel R, and Netea MG. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol.* 2014;155(2):213-9.
13. Hole CR, Wager CML, Castro-Lopez N, Campuzano A, Cai H, Wozniak KL, Wang Y, and Wormley FL, Jr. Induction of memory-like dendritic cell responses in vivo. *Nat Commun.* 2019;10(1):2955.
14. Ebihara T, Tatematsu M, Fuchimukai A, Yamada T, Yamagata K, Takasuga S, and Yamada T. Trained innate lymphoid cells in allergic diseases. *Allergol Int.* 2021;70(2):174-80.
15. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonca LE, Pacis A, Tzelepis F, Pernet E, Dumaine A, Grenier JC, et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. *Cell.* 2018;172(1-2):176-90 e19.
16. Chavakis T, Mitroulis I, and Hajishengallis G. Hematopoietic progenitor cells as integrative hubs for adaptation to and fine-tuning of inflammation. *Nat Immunol.* 2019;20(7):802-11.
17. Fanucchi S, Dominguez-Andres J, Joosten LAB, Netea MG, and Mhlanga MM. The Intersection of Epigenetics and Metabolism in Trained Immunity. *Immunity.* 2021;54(1):32-43.
18. Arts RJW, Moorlag S, Novakovic B, Li Y, Wang SY, Oosting M, Kumar V, Xavier RJ, Wijmenga C, Joosten LAB, et al. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host Microbe.* 2018;23(1):89-100 e5.
19. Brandi P, Conejero L, Cueto FJ, Martinez-Cano S, Dunphy G, Gomez MJ, Relano C, Saz-Leal P, Enamorado M, Quintas A, et al. Trained immunity induction by the inactivated mucosal vaccine MV130 protects against experimental viral respiratory infections. *Cell Rep.* 2022;38(1):110184.
20. Martin-Cruz L, Sevilla-Ortega C, Angelina A, Dominguez-Andres J, Netea MG, Subiza JL, and Palomares O. From trained immunity in allergy to trained immunity-based allergen vaccines. *Clin Exp Allergy.* 2022.
21. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, and Sancho D. Dendritic cells in cancer immunology and immunotherapy. *Nat Rev Immunol.* 2020;20(1):7-24.
22. Palomares O, O'Mahony L, and Akdis CA. The many routes of dendritic cells to ensure immune regulation. *J Allergy Clin Immunol.* 2011;127(6):1541-2.

23. Steinman RM, and Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med.* 1973;137(5):1142-62.
24. Collin M, and Bigley V. Human dendritic cell subsets: an update. *Immunology.* 2018;154(1):3-20.
25. Novak N, and Bieber T. 2. Dendritic cells as regulators of immunity and tolerance. *J Allergy Clin Immunol.* 2008;121(2 Suppl):S370-4; quiz S413.
26. Fucikova J, Palova-Jelinkova L, Bartunkova J, and Spisek R. Induction of Tolerance and Immunity by Dendritic Cells: Mechanisms and Clinical Applications. *Front Immunol.* 2019;10(2393.
27. Mempel TR, Henrickson SE, and Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature.* 2004;427(6970):154-9.
28. Hilligan KL, and Ronchese F. Antigen presentation by dendritic cells and their instruction of CD4+ T helper cell responses. *Cell Mol Immunol.* 2020;17(6):587-99.
29. de Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhsh M, and Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. *J Immunol.* 2002;168(4):1704-9.
30. Merad M, Sathe P, Helft J, Miller J, and Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol.* 2013;31(563-604.
31. Askmyr D, Abolhalaj M, Gomez Jimenez D, Greiff L, Lindstedt M, and Lundberg K. Pattern recognition receptor expression and maturation profile of dendritic cell subtypes in human tonsils and lymph nodes. *Hum Immunol.* 2021;82(12):976-81.
32. Lambrecht BN. Allergen uptake and presentation by dendritic cells. *Curr Opin Allergy Clin Immunol.* 2001;1(1):51-9.
33. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, Kaisho T, Bogunovic D, Bhardwaj N, and Krummel MF. Critical Role for CD103(+)/CD141(+) Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma. *Cancer Cell.* 2016;30(2):324-36.
34. Walsh KP, and Mills KH. Dendritic cells and other innate determinants of T helper cell polarisation. *Trends Immunol.* 2013;34(11):521-30.
35. Chiang MC, Tullett KM, Lee YS, Idris A, Ding Y, McDonald KJ, Kassianos A, Leal Rojas IM, Jeet V, Lahoud MH, et al. Differential uptake and cross-presentation of

soluble and necrotic cell antigen by human DC subsets. *Eur J Immunol.* 2016;46(2):329-39.

36. Gros M, and Amigorena S. Regulation of Antigen Export to the Cytosol During Cross-Presentation. *Front Immunol.* 2019;10(41.
37. Palomares O, Martin-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, and Akdis CA. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. *Genes Immun.* 2014;15(8):511-20.
38. Sirvent S, Soria I, Cirauqui C, Cases B, Manzano AI, Diez-Rivero CM, Reche PA, Lopez-Relano J, Martinez-Naves E, Canada FJ, et al. Novel vaccines targeting dendritic cells by coupling allergoids to nonoxidized mannan enhance allergen uptake and induce functional regulatory T cells through programmed death ligand 1. *J Allergy Clin Immunol.* 2016;138(2):558-67 e11.
39. Palomares O, Ruckert B, Jartti T, Kucuksezer UC, Puhakka T, Gomez E, Fahrner HB, Speiser A, Jung A, Kwok WW, et al. Induction and maintenance of allergen-specific FOXP3+ Treg cells in human tonsils as potential first-line organs of oral tolerance. *J Allergy Clin Immunol.* 2012;129(2):510-20, 20 e1-9.
40. Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, Qin XF, Liu YJ, and Gilliet M. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med.* 2007;204(1):105-15.
41. Kawai T, and Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* 2010;11(5):373-84.
42. Iborra S, and Sancho D. Signalling versatility following self and non-self sensing by myeloid C-type lectin receptors. *Immunobiology.* 2015;220(2):175-84.
43. Kawai T, and Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity.* 2011;34(5):637-50.
44. Fitzgerald KA, and Kagan JC. Toll-like Receptors and the Control of Immunity. *Cell.* 2020;180(6):1044-66.
45. Kim YK, Shin JS, and Nahm MH. NOD-Like Receptors in Infection, Immunity, and Diseases. *Yonsei Med J.* 2016;57(1):5-14.
46. Platnich JM, and Muruve DA. NOD-like receptors and inflammasomes: A review of their canonical and non-canonical signaling pathways. *Arch Biochem Biophys.* 2019;670(4-14.
47. Rehwinkel J, and Gack MU. RIG-I-like receptors: their regulation and roles in RNA sensing. *Nat Rev Immunol.* 2020;20(9):537-51.
48. Loo YM, and Gale M, Jr. Immune signaling by RIG-I-like receptors. *Immunity.* 2011;34(5):680-92.

49. Busold S, Nagy NA, Tas SW, van Ree R, de Jong EC, and Geijtenbeek TBH. Various Tastes of Sugar: The Potential of Glycosylation in Targeting and Modulating Human Immunity via C-Type Lectin Receptors. *Front Immunol.* 2020;11(134.
50. Sancho D, and Reis e Sousa C. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu Rev Immunol.* 2012;30(491-529.
51. Geijtenbeek TB, and Gringhuis SI. C-type lectin receptors in the control of T helper cell differentiation. *Nat Rev Immunol.* 2016;16(7):433-48.
52. Del Fresno C, Iborra S, Saz-Leal P, Martinez-Lopez M, and Sancho D. Flexible Signaling of Myeloid C-Type Lectin Receptors in Immunity and Inflammation. *Front Immunol.* 2018;9(804.
53. Kerrigan AM, and Brown GD. Syk-coupled C-type lectins in immunity. *Trends Immunol.* 2011;32(4):151-6.
54. Watowich SS, and Liu YJ. Mechanisms regulating dendritic cell specification and development. *Immunol Rev.* 2010;238(1):76-92.
55. Eisenbarth SC. Dendritic cell subsets in T cell programming: location dictates function. *Nat Rev Immunol.* 2019;19(2):89-103.
56. Anderson DA, 3rd, Dutertre CA, Ginhoux F, and Murphy KM. Genetic models of human and mouse dendritic cell development and function. *Nat Rev Immunol.* 2021;21(2):101-15.
57. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, Griesbeck M, Butler A, Zheng S, Lazo S, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science.* 2017;356(6335).
58. Guilliams M, Ginhoux F, Jakubzick C, Naik SH, Onai N, Schraml BU, Segura E, Tussiwand R, and Yona S. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol.* 2014;14(8):571-8.
59. Segura E. Human dendritic cell subsets: An updated view of their ontogeny and functional specialization. *Eur J Immunol.* 2022;52(11):1759-67.
60. Dutertre CA, Becht E, Irac SE, Khalilnezhad A, Narang V, Khalilnezhad S, Ng PY, van den Hoogen LL, Leong JY, Lee B, et al. Single-Cell Analysis of Human Mononuclear Phagocytes Reveals Subset-Defining Markers and Identifies Circulating Inflammatory Dendritic Cells. *Immunity.* 2019;51(3):573-89 e8.
61. Cytlak U, Resteu A, Pagan S, Green K, Milne P, Maisuria S, McDonald D, Hulme G, Filby A, Carpenter B, et al. Differential IRF8 Transcription Factor Requirement Defines Two Pathways of Dendritic Cell Development in Humans. *Immunity.* 2020;53(2):353-70 e8.

62. Guilliams M, Dutertre CA, Scott CL, McGovern N, Sichien D, Chakarov S, Van Gassen S, Chen J, Poidinger M, De Prijck S, et al. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity.* 2016;45(3):669-84.
63. Macri C, Pang ES, Patton T, and O'Keeffe M. Dendritic cell subsets. *Semin Cell Dev Biol.* 2018;84(11-21.
64. Noubade R, Majri-Morrison S, and Tarbell KV. Beyond cDC1: Emerging Roles of DC Crosstalk in Cancer Immunity. *Front Immunol.* 2019;10(1014.
65. Amon L, Lehmann CHK, Heger L, Heidkamp GF, and Dudziak D. The ontogenetic path of human dendritic cells. *Mol Immunol.* 2020;120(122-9.
66. Saito Y, Komori S, Kotani T, Murata Y, and Matozaki T. The Role of Type-2 Conventional Dendritic Cells in the Regulation of Tumor Immunity. *Cancers (Basel).* 2022;14(8).
67. Leylek R, Alcantara-Hernandez M, Lanzar Z, Ludtke A, Perez OA, Reizis B, and Idoyaga J. Integrated Cross-Species Analysis Identifies a Conserved Transitional Dendritic Cell Population. *Cell Rep.* 2019;29(11):3736-50 e8.
68. Swiecki M, and Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol.* 2015;15(8):471-85.
69. Reizis B. Plasmacytoid Dendritic Cells: Development, Regulation, and Function. *Immunity.* 2019;50(1):37-50.
70. Lopez-Abente J, Benito-Villalvilla C, Jaumont X, Pfister P, Tassinari P, and Palomares O. Omalizumab restores the ability of human plasmacytoid dendritic cells to induce Foxp3(+)Tregs. *Eur Respir J.* 2021;57(1).
71. Martin-Gayo E, Sierra-Filardi E, Corbi AL, and Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood.* 2010;115(26):5366-75.
72. Dominguez PM, and Ardavin C. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. *Immunol Rev.* 2010;234(1):90-104.
73. Morante-Palacios O, Fondelli F, Ballestar E, and Martinez-Caceres EM. Tolerogenic Dendritic Cells in Autoimmunity and Inflammatory Diseases. *Trends Immunol.* 2021;42(1):59-75.
74. Del Prete A, Sozio F, Barbazza I, Salvi V, Tiberio L, Laffranchi M, Gismondi A, Bosisio D, Schioppa T, and Sozzani S. Functional Role of Dendritic Cell Subsets in Cancer Progression and Clinical Implications. *Int J Mol Sci.* 2020;21(11).

75. Plesca I, Muller L, Bottcher JP, Medyouf H, Wehner R, and Schmitz M. Tumor-associated human dendritic cell subsets: Phenotype, functional orientation, and clinical relevance. *Eur J Immunol.* 2022;52(11):1750-8.
76. Posch W, Lass-Florl C, and Wilflingseder D. Generation of Human Monocyte-derived Dendritic Cells from Whole Blood. *J Vis Exp.* 2016118).
77. Sallusto F, and Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 1994;179(4):1109-18.
78. Benito-Villalvilla C, Cirauqui C, Diez-Rivero CM, Casanovas M, Subiza JL, and Palomares O. MV140, a sublingual polyvalent bacterial preparation to treat recurrent urinary tract infections, licenses human dendritic cells for generating Th1, Th17, and IL-10 responses via Syk and MyD88. *Mucosal Immunol.* 2017;10(4):924-35.
79. Cirauqui C, Benito-Villalvilla C, Sanchez-Ramon S, Sirvent S, Diez-Rivero CM, Conejero L, Brandi P, Hernandez-Cillero L, Ochoa JL, Perez-Villamil B, et al. Human dendritic cells activated with MV130 induce Th1, Th17 and IL-10 responses via RIPK2 and MyD88 signalling pathways. *Eur J Immunol.* 2018;48(1):180-93.
80. Angelina A, Perez-Diego M, Lopez-Abente J, Ruckert B, Nombela I, Akdis M, Martin-Fontecha M, Akdis C, and Palomares O. Cannabinoids induce functional Tregs by promoting tolerogenic DCs via autophagy and metabolic reprograming. *Mucosal Immunol.* 2022;15(1):96-108.
81. Benito-Villalvilla C, Perez-Diego M, Angelina A, Kisand K, Rebane A, Subiza JL, and Palomares O. Allergoid-mannan conjugates reprogram monocytes into tolerogenic dendritic cells via epigenetic and metabolic rewiring. *J Allergy Clin Immunol.* 2022;149(1):212-22 e9.
82. Spary LK, Salimu J, Webber JP, Clayton A, Mason MD, and Tabi Z. Tumor stroma-derived factors skew monocyte to dendritic cell differentiation toward a suppressive CD14(+) PD-L1(+) phenotype in prostate cancer. *Oncoimmunology.* 2014;3(9):e955331.
83. Esensten JH, Helou YA, Chopra G, Weiss A, and Bluestone JA. CD28 Costimulation: From Mechanism to Therapy. *Immunity.* 2016;44(5):973-88.
84. Peng Q, Qiu X, Zhang Z, Zhang S, Zhang Y, Liang Y, Guo J, Peng H, Chen M, Fu YX, et al. PD-L1 on dendritic cells attenuates T cell activation and regulates response to immune checkpoint blockade. *Nat Commun.* 2020;11(1):4835.

85. Rowshanravan B, Halliday N, and Sansom DM. CTLA-4: a moving target in immunotherapy. *Blood.* 2018;131(1):58-67.
86. Munn DH, and Mellor AL. IDO in the Tumor Microenvironment: Inflammation, Counter-Regulation, and Tolerance. *Trends Immunol.* 2016;37(3):193-207.
87. DuPage M, and Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol.* 2016;16(3):149-63.
88. Zhu J. T Helper Cell Differentiation, Heterogeneity, and Plasticity. *Cold Spring Harb Perspect Biol.* 2018;10(10).
89. Maggi E, Parronchi P, Azzarone BG, and Moretta L. A pathogenic integrated view explaining the different endotypes of asthma and allergic disorders. *Allergy.* 2022.
90. Sallusto F. Heterogeneity of Human CD4(+) T Cells Against Microbes. *Annu Rev Immunol.* 2016;34(317-34.
91. Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, Duan S, Eiwegger T, Eljaszewicz A, Ferstl R, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor beta, and TNF-alpha: Receptors, functions, and roles in diseases. *J Allergy Clin Immunol.* 2016;138(4):984-1010.
92. Saravia J, Chapman NM, and Chi H. Helper T cell differentiation. *Cell Mol Immunol.* 2019;16(7):634-43.
93. Kaplan MH, Hufford MM, and Olson MR. The development and in vivo function of T helper 9 cells. *Nat Rev Immunol.* 2015;15(5):295-307.
94. Doulabi H, Masoumi E, Rastin M, Foolady Azarnaminy A, Esmaeili SA, and Mahmoudi M. The role of Th22 cells, from tissue repair to cancer progression. *Cytokine.* 2022;149(155749.
95. Song W, and Craft J. T follicular helper cell heterogeneity: Time, space, and function. *Immunol Rev.* 2019;288(1):85-96.
96. Shamji MH, Sharif H, Layhadi JA, Zhu R, Kishore U, and Renz H. Diverse immune mechanisms of allergen immunotherapy for allergic rhinitis with and without asthma. *J Allergy Clin Immunol.* 2022;149(3):791-801.
97. Sharif H, Acharya S, Dhondalay GKR, Varricchi G, Krasner-Macleod S, Laisuan W, Switzer A, Lenormand M, Kashe E, Parkin RV, et al. Altered chromatin landscape in circulating T follicular helper and regulatory cells following grass pollen subcutaneous and sublingual immunotherapy. *J Allergy Clin Immunol.* 2021;147(2):663-76.
98. Wing JB, Tanaka A, and Sakaguchi S. Human FOXP3(+) Regulatory T Cell Heterogeneity and Function in Autoimmunity and Cancer. *Immunity.* 2019;50(2):302-16.

99. Palomares O, Akdis M, Martin-Fontecha M, and Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunol Rev.* 2017;278(1):219-36.
100. Plitas G, and Rudensky AY. Regulatory T Cells: Differentiation and Function. *Cancer Immunol Res.* 2016;4(9):721-5.
101. Palomares O, Elewaut D, Irving PM, Jaumont X, and Tassinari P. Regulatory T cells and immunoglobulin E: A new therapeutic link for autoimmunity? *Allergy.* 2022;77(11):3293-308.
102. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, et al. Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol.* 2013;14(4):307-8.
103. Hori S, Nomura T, and Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003;299(5609):1057-61.
104. Glasner A, and Plitas G. Tumor resident regulatory T cells. *Semin Immunol.* 2021;52(101476.
105. Lucca LE, and Dominguez-Villar M. Modulation of regulatory T cell function and stability by co-inhibitory receptors. *Nat Rev Immunol.* 2020;20(11):680-93.
106. O'Neill LA, Kishton RJ, and Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol.* 2016;16(9):553-65.
107. Wculek SK, Khouili SC, Priego E, Heras-Murillo I, and Sancho D. Metabolic Control of Dendritic Cell Functions: Digesting Information. *Front Immunol.* 2019;10(775.
108. O'Neill LA, and Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med.* 2016;213(1):15-23.
109. Pearce EJ, and Everts B. Dendritic cell metabolism. *Nat Rev Immunol.* 2015;15(1):18-29.
110. Benito-Villalvilla C, Soria I, Perez-Diego M, Fernandez-Caldas E, Subiza JL, and Palomares O. Alum impairs tolerogenic properties induced by allergoid-mannan conjugates inhibiting mTOR and metabolic reprogramming in human DCs. *Allergy.* 2020;75(3):648-59.
111. Romero-Garcia S, Moreno-Altamirano MM, Prado-Garcia H, and Sanchez-Garcia FJ. Lactate Contribution to the Tumor Microenvironment: Mechanisms, Effects on Immune Cells and Therapeutic Relevance. *Front Immunol.* 2016;7(52.
112. Krawczyk CM, Holowka T, Sun J, Blagih J, Amiel E, DeBerardinis RJ, Cross JR, Jung E, Thompson CB, Jones RG, et al. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood.* 2010;115(23):4742-9.

113. Ryan DG, and O'Neill LAJ. Krebs cycle rewired for macrophage and dendritic cell effector functions. *FEBS Lett.* 2017;591(19):2992-3006.
114. Snyder JP, and Amiel E. Regulation of Dendritic Cell Immune Function and Metabolism by Cellular Nutrient Sensor Mammalian Target of Rapamycin (mTOR). *Front Immunol.* 2018;9(3145.
115. Malinarich F, Duan K, Hamid RA, Bijin A, Lin WX, Poidinger M, Fairhurst AM, and Connolly JE. High mitochondrial respiration and glycolytic capacity represent a metabolic phenotype of human tolerogenic dendritic cells. *J Immunol.* 2015;194(11):5174-86.
116. Sim WJ, Ahl PJ, and Connolly JE. Metabolism Is Central to Tolerogenic Dendritic Cell Function. *Mediators Inflamm.* 2016;2016(2636701.
117. Marin E, Bouchet-Delbos L, Renoult O, Louvet C, Nerriere-Daguin V, Managh AJ, Even A, Giraud M, Vu Manh TP, Aguesse A, et al. Human Tolerogenic Dendritic Cells Regulate Immune Responses through Lactate Synthesis. *Cell Metab.* 2019;30(6):1075-90 e8.
118. Dominguez-Andres J, Joosten LA, and Netea MG. Induction of innate immune memory: the role of cellular metabolism. *Curr Opin Immunol.* 2019;56(10-6.
119. Riksen NP, and Netea MG. Immunometabolic control of trained immunity. *Mol Aspects Med.* 2021;77(100897.
120. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, Giamarellos-Bourboulis EJ, Martens JH, Rao NA, Aghajanirefah A, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science.* 2014;345(6204):1250684.
121. Arts RJ, Novakovic B, Ter Horst R, Carvalho A, Bekkering S, Lachmandas E, Rodrigues F, Silvestre R, Cheng SC, Wang SY, et al. Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. *Cell Metab.* 2016;24(6):807-19.
122. Arts RJW, Carvalho A, La Rocca C, Palma C, Rodrigues F, Silvestre R, Kleinnijenhuis J, Lachmandas E, Goncalves LG, Belinha A, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. *Cell Rep.* 2016;17(10):2562-71.
123. Dominguez-Andres J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, Oosting M, Moorlag S, Groh LA, Zwaag J, et al. The Itaconate Pathway Is a Central Regulatory Node Linking Innate Immune Tolerance and Trained Immunity. *Cell Metab.* 2019;29(1):211-20 e5.

124. Bekkering S, Arts RJW, Novakovic B, Kourtzelis I, van der Heijden C, Li Y, Popa CD, Ter Horst R, van Tuijl J, Netea-Maier RT, et al. Metabolic Induction of Trained Immunity through the Mevalonate Pathway. *Cell.* 2018;172(1-2):135-46 e9.
125. Etchegaray JP, and Mostoslavsky R. Interplay between Metabolism and Epigenetics: A Nuclear Adaptation to Environmental Changes. *Mol Cell.* 2016;62(5):695-711.
126. Allis CD, and Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet.* 2016;17(8):487-500.
127. Mazzone R, Zwergel C, Artico M, Taurone S, Ralli M, Greco A, and Mai A. The emerging role of epigenetics in human autoimmune disorders. *Clin Epigenetics.* 2019;11(1):34.
128. Lawrence M, Daujat S, and Schneider R. Lateral Thinking: How Histone Modifications Regulate Gene Expression. *Trends Genet.* 2016;32(1):42-56.
129. Kouzarides T. Chromatin modifications and their function. *Cell.* 2007;128(4):693-705.
130. Kimura H. Histone modifications for human epigenome analysis. *J Hum Genet.* 2013;58(7):439-45.
131. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, Cheng SC, Ratter J, Berentsen K, van der Ent MA, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science.* 2014;345(6204):1251086.
132. Flores-Mireles AL, Walker JN, Caparon M, and Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nat Rev Microbiol.* 2015;13(5):269-84.
133. Denning DW, Kneale M, Sobel JD, and Rautemaa-Richardson R. Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis.* 2018;18(11):e339-e47.
134. Foxman B. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect Dis Clin North Am.* 2014;28(1):1-13.
135. Butler AM, Durkin MJ, Keller MR, Ma Y, Powderly WG, and Olsen MA. Association of Adverse Events With Antibiotic Treatment for Urinary Tract Infection. *Clin Infect Dis.* 2022;74(8):1408-18.
136. Fisher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, Bromley M, Bruggemann R, Garber G, Cornely OA, et al. Tackling the emerging threat of antifungal resistance to human health. *Nat Rev Microbiol.* 2022;20(9):557-71.

137. Shukla A, and Sobel JD. Vulvovaginitis Caused by Candida Species Following Antibiotic Exposure. *Curr Infect Dis Rep.* 2019;21(11):44.
138. Marchaim D, Lemanek L, Bheemreddy S, Kaye KS, and Sobel JD. Fluconazole-resistant Candida albicans vulvovaginitis. *Obstet Gynecol.* 2012;120(6):1407-14.
139. Foxman B. The epidemiology of urinary tract infection. *Nat Rev Urol.* 2010;7(12):653-60.
140. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Dis Mon.* 2003;49(2):53-70.
141. Medina M, and Castillo-Pino E. An introduction to the epidemiology and burden of urinary tract infections. *Ther Adv Urol.* 2019;11(1756287219832172.
142. Wagenlehner FME, Bjerklund Johansen TE, Cai T, Koves B, Kranz J, Pilatz A, and Tandogdu Z. Epidemiology, definition and treatment of complicated urinary tract infections. *Nat Rev Urol.* 2020;17(10):586-600.
143. Klein RD, and Hultgren SJ. Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. *Nat Rev Microbiol.* 2020;18(4):211-26.
144. Goossens H, Ferech M, Vander Stichele R, Elseviers M, and Group EP. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet.* 2005;365(9459):579-87.
145. Costelloe C, Metcalfe C, Lovering A, Mant D, and Hay AD. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: systematic review and meta-analysis. *BMJ.* 2010;340(c2096.
146. Stracy M, Snitser O, Yelin I, Amer Y, Parizade M, Katz R, Rimler G, Wolf T, Herzel E, Koren G, et al. Minimizing treatment-induced emergence of antibiotic resistance in bacterial infections. *Science.* 2022;375(6583):889-94.
147. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance. Accessed December 12, 2022.
148. Antimicrobial Resistance C. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet.* 2022;399(10325):629-55.
149. Elvers KT, Wilson VJ, Hammond A, Duncan L, Huntley AL, Hay AD, and van der Werf ET. Antibiotic-induced changes in the human gut microbiota for the most commonly prescribed antibiotics in primary care in the UK: a systematic review. *BMJ Open.* 2020;10(9):e035677.
150. Butler D, Ambite I, Wan MLY, Tran TH, Wullt B, and Svanborg C. Immunomodulation therapy offers new molecular strategies to treat UTI. *Nat Rev Urol.* 2022;19(7):419-37.

151. Wawrysiuk S, Naber K, Rechberger T, and Miotla P. Prevention and treatment of uncomplicated lower urinary tract infections in the era of increasing antimicrobial resistance-non-antibiotic approaches: a systemic review. *Arch Gynecol Obstet.* 2019;300(4):821-8.
152. Lorenzo-Gómez MF, Foley S, Nickel JC, García-Cenador MB, Padilla-Fernández BY, Casado IG, Martínez-Huélamo M, Yang B, Blick C, Ferreira F, et al. Sublingual MV140 for Prevention of Recurrent Urinary Tract Infections. *NEJM Evidence.* 2022;1(4):EVIDoa2100018.
153. Brodie A, El-Taji O, Jour I, Foley C, and Hanbury D. A Retrospective Study of Immunotherapy Treatment with Uro-Vaxom (OM-89(R)) for Prophylaxis of Recurrent Urinary Tract Infections. *Curr Urol.* 2020;14(3):130-4.
154. Mydock-McGrane L, Cusumano Z, Han Z, Binkley J, Kostakioti M, Hannan T, Pinkner JS, Klein R, Kalas V, Crowley J, et al. Antivirulence C-Mannosides as Antibiotic-Sparing, Oral Therapeutics for Urinary Tract Infections. *J Med Chem.* 2016;59(20):9390-408.
155. Sobel JD. Vulvovaginal candidosis. *Lancet.* 2007;369(9577):1961-71.
156. Foxman B, Muraglia R, Dietz JP, Sobel JD, and Wagner J. Prevalence of recurrent vulvovaginal candidiasis in 5 European countries and the United States: results from an internet panel survey. *J Low Genit Tract Dis.* 2013;17(3):340-5.
157. Sobel JD. Recurrent vulvovaginal candidiasis. *Am J Obstet Gynecol.* 2016;214(1):15-21.
158. Ilkit M, and Guzel AB. The epidemiology, pathogenesis, and diagnosis of vulvovaginal candidosis: a mycological perspective. *Crit Rev Microbiol.* 2011;37(3):250-61.
159. Kennedy MA, and Sobel JD. Vulvovaginal Candidiasis Caused by Non-albicans Candida Species: New Insights. *Curr Infect Dis Rep.* 2010;12(6):465-70.
160. Xu J, Schwartz K, Bartoces M, Monsur J, Severson RK, and Sobel JD. Effect of antibiotics on vulvovaginal candidiasis: a MetroNet study. *J Am Board Fam Med.* 2008;21(4):261-8.
161. Pirotta MV, and Garland SM. Genital Candida species detected in samples from women in Melbourne, Australia, before and after treatment with antibiotics. *J Clin Microbiol.* 2006;44(9):3213-7.
162. Tachedjian G, Aldunate M, Bradshaw CS, and Cone RA. The role of lactic acid production by probiotic Lactobacillus species in vaginal health. *Res Microbiol.* 2017;168(9-10):782-92.
163. Stapleton AE. The Vaginal Microbiota and Urinary Tract Infection. *Microbiol Spectr.* 2016;4(6).

164. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, et al. Clinical Practice Guideline for the Management of Candidiasis: 2016 Update by the Infectious Diseases Society of America. *Clin Infect Dis.* 2016;62(4):e1-50.

165. Sherrard J, Wilson J, Donders G, Mendling W, and Jensen JS. 2018 European (IUSTI/WHO) International Union against sexually transmitted infections (IUSTI) World Health Organisation (WHO) guideline on the management of vaginal discharge. *Int J STD AIDS.* 2018;29(13):1258-72.

166. Crouss T, Sobel JD, Smith K, and Nyirjesy P. Long-Term Outcomes of Women With Recurrent Vulvovaginal Candidiasis After a Course of Maintenance Antifungal Therapy. *J Low Genit Tract Dis.* 2018;22(4):382-6.

167. Brandolt TM, Klafke GB, Goncalves CV, Bitencourt LR, Martinez AM, Mendes JF, Meireles MC, and Xavier MO. Prevalence of Candida spp. in cervical-vaginal samples and the in vitro susceptibility of isolates. *Braz J Microbiol.* 2017;48(1):145-50.

168. De Bernardis F, Graziani S, Tirelli F, and Antonopoulou S. Candida vaginitis: virulence, host response and vaccine prospects. *Med Mycol.* 2018;56(suppl_1):26-31.

169. Oliveira LVN, Wang R, Specht CA, and Levitz SM. Vaccines for human fungal diseases: close but still a long way to go. *NPJ Vaccines.* 2021;6(1):33.

170. Edwards JE, Jr., Schwartz MM, Schmidt CS, Sobel JD, Nyirjesy P, Schodel F, Marchus E, Lizakowski M, DeMontigny EA, Hoeg J, et al. A Fungal Immunotherapeutic Vaccine (NDV-3A) for Treatment of Recurrent Vulvovaginal Candidiasis-A Phase 2 Randomized, Double-Blind, Placebo-Controlled Trial. *Clin Infect Dis.* 2018;66(12):1928-36.

171. Tomas M, Rolo J, Gaspar C, Palmeira-de-Oliveira A, Simoes S, Katz DF, Martinez-de-Oliveira J, and Palmeira-de-Oliveira R. Sodium bicarbonate gels: a new promising strategy for the treatment of vulvovaginal candidosis. *Eur J Pharm Sci.* 2021;157(105621.

172. Dominguez-Andres J, van Crevel R, Divangahi M, and Netea MG. Designing the Next Generation of Vaccines: Relevance for Future Pandemics. *mBio.* 2020;11(6).

173. Sanchez-Ramon S, Conejero L, Netea MG, Sancho D, Palomares O, and Subiza JL. Trained Immunity-Based Vaccines: A New Paradigm for the Development of Broad-Spectrum Anti-infectious Formulations. *Front Immunol.* 2018;9(2936.

174. Boyd SD, and Jackson KJL. Predicting vaccine responsiveness. *Cell Host Microbe.* 2015;17(3):301-7.

175. Benn CS, Fisker AB, Rieckmann A, Sorup S, and Aaby P. Vaccinology: time to change the paradigm? *Lancet Infect Dis.* 2020;20(10):e274-e83.
176. Netea MG, and van Crevel R. BCG-induced protection: effects on innate immune memory. *Semin Immunol.* 2014;26(6):512-7.
177. Giamarellos-Bourboulis EJ, Tsilika M, Moorlag S, Antonakos N, Kotsaki A, Dominguez-Andres J, Kyriazopoulou E, Gkavogianni T, Adami ME, Damoraki G, et al. Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly. *Cell.* 2020;183(2):315-23 e9.
178. Lee YJ, Lee JY, Jang YH, Seo SU, Chang J, and Seong BL. Non-specific Effect of Vaccines: Immediate Protection against Respiratory Syncytial Virus Infection by a Live Attenuated Influenza Vaccine. *Front Microbiol.* 2018;9(83.
179. Piedra PA, Gaglani MJ, Kozinetz CA, Herschler GB, Fewlass C, Harvey D, Zimmerman N, and Glezen WP. Trivalent live attenuated intranasal influenza vaccine administered during the 2003-2004 influenza type A (H3N2) outbreak provided immediate, direct, and indirect protection in children. *Pediatrics.* 2007;120(3):e553-64.
180. Gillard GO, Bivas-Benita M, Hovav AH, Grandpre LE, Panas MW, Seaman MS, Haynes BF, and Letvin NL. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. *PLoS Pathog.* 2011;7(8):e1002141.
181. Nielsen S, Sujan HM, Benn CS, Aaby P, and Hanifi SMA. Oral Polio Vaccine Campaigns May Reduce the Risk of Death from Respiratory Infections. *Vaccines (Basel).* 2021;9(10).
182. Ovsyannikova IG, Reid KC, Jacobson RM, Oberg AL, Klee GG, and Poland GA. Cytokine production patterns and antibody response to measles vaccine. *Vaccine.* 2003;21(25-26):3946-53.
183. Tejera-Alhambra M, Palomares O, Perez de Diego R, Diaz-Lezcano I, and Sanchez-Ramon S. New Biological Insights in the Immunomodulatory Effects of Mucosal Polybacterial Vaccines in Clinical Practice. *Curr Pharm Des.* 2016;22(41):6283-93.
184. Lavelle EC, and Ward RW. Mucosal vaccines - fortifying the frontiers. *Nat Rev Immunol.* 2022;22(4):236-50.
185. Holmgren J, and Czerkinsky C. Mucosal immunity and vaccines. *Nat Med.* 2005;11(4 Suppl):S45-53.
186. Negri DR, Riccomi A, Pinto D, Vendetti S, Rossi A, Cicconi R, Ruggiero P, Del Giudice G, and De Magistris MT. Persistence of mucosal and systemic immune responses following sublingual immunization. *Vaccine.* 2010;28(25):4175-80.

187. Sanchez-Ramon S, Perez de Diego R, Dieli-Crimi R, and Subiza JL. Extending the clinical horizons of mucosal bacterial vaccines: current evidence and future prospects. *Curr Drug Targets.* 2014;15(12):1132-43.
188. Vierboom MPM, Dijkman K, Sombroek CC, Hofman SO, Boot C, Vervenne RAW, Haanstra KG, van der Sande M, van Emst L, Dominguez-Andres J, et al. Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination. *Cell Rep Med.* 2021;2(1):100185.
189. Lycke N. Recent progress in mucosal vaccine development: potential and limitations. *Nat Rev Immunol.* 2012;12(8):592-605.
190. Loubet P, Ranfaing J, Dinh A, Dunyach-Remy C, Bernard L, Bruyere F, Lavigne JP, and Sotto A. Alternative Therapeutic Options to Antibiotics for the Treatment of Urinary Tract Infections. *Front Microbiol.* 2020;11(1509.
191. Andreu A, Planells I, and Grupo Cooperativo Espanol para el Estudio de la Sensibilidad Antimicrobiana de los Patogenos U. [Etiology of community-acquired lower urinary infections and antimicrobial resistance of Escherichia coli: a national surveillance study]. *Med Clin (Barc).* 2008;130(13):481-6.
192. Carrion-Lopez P, Martinez-Ruiz J, Libran-Garcia L, Gimenez-Bachs JM, Pastor-Navarro H, and Salinas-Sanchez AS. Analysis of the Efficacy of a Sublingual Bacterial Vaccine in the Prophylaxis of Recurrent Urinary Tract Infection. *Urol Int.* 2020;104(3-4):293-300.
193. Ramirez Sevilla C, Gomez Lanza E, Manzanera JL, Martin JAR, and Sanz MAB. Active immunoprophyilaxis with uromune(R) decreases the recurrence of urinary tract infections at three and six months after treatment without relevant secondary effects. *BMC Infect Dis.* 2019;19(1):901.
194. Yang B, and Foley S. First experience in the UK of treating women with recurrent urinary tract infections with the bacterial vaccine Uromune((R)). *BJU Int.* 2018;121(2):289-92.
195. Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Cenador MB, Virseda-Rodriguez AJ, Martin-Garcia I, Sanchez-Escudero A, Vicente-Arroyo MJ, and Miron-Canelo JA. Comparison of sublingual therapeutic vaccine with antibiotics for the prophylaxis of recurrent urinary tract infections. *Front Cell Infect Microbiol.* 2015;5(50.
196. Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Criado FJ, Miron-Canelo JA, Gil-Vicente A, Nieto-Huertos A, and Silva-Abuin JM. Evaluation of a therapeutic vaccine for the prevention of recurrent urinary tract infections versus prophylactic treatment with antibiotics. *Int Urogynecol J.* 2013;24(1):127-34.

197. Lorenzo-Gomez MF, Padilla-Fernandez B, Flores-Fraile J, Valverde-Martinez S, Gonzalez-Casado I, Hernandez JD, Sanchez-Escudero A, Vicente Arroyo MJ, Martinez-Huelamo M, Criado FH, et al. Impact of whole-cell bacterial immunoprophylaxis in the management of recurrent urinary tract infections in the frail elderly. *Vaccine.* 2021;39(42):6308-14.

198. Sanchez-Ramon S, Fernandez-Paredes L, Saz-Leal P, Diez-Rivero CM, Ochoa-Grullon J, Morado C, Macarron P, Martinez C, Villaverde V, de la Pena AR, et al. Sublingual Bacterial Vaccination Reduces Recurrent Infections in Patients With Autoimmune Diseases Under Immunosuppressant Treatment. *Front Immunol.* 2021;12(675735.

199. Carrion-Lopez P, Martinez-Ruiz J, Gimenez-Bachs JM, Fernandez-Anguita PJ, Diaz de Mera-Sanchez Migallon I, Legido-Gomez O, Rico-Marco S, Lorenzo-Sanchez MV, and Salinas-Sanchez AS. Cost-Effectiveness of a Sublingual Bacterial Vaccine for the Prophylaxis of Recurrent Urinary Tract Infections. *Urol Int.* 2022;106(7):730-6.

200. Tso GHW, Reales-Calderon JA, and Pavelka N. The Elusive Anti-Candida Vaccine: Lessons From the Past and Opportunities for the Future. *Front Immunol.* 2018;9(897.

201. De Bernardis F, Arancia S, Sandini S, Graziani S, and Norelli S. Studies of Immune Responses in Candida vaginitis. *Pathogens.* 2015;4(4):697-707.

202. https://www.who.int/health-topics/cancer#tab=tab_1. Accessed December 12, 2022.

203. Lambert AW, Pattabiraman DR, and Weinberg RA. Emerging Biological Principles of Metastasis. *Cell.* 2017;168(4):670-91.

204. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, and Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin.* 2021;71(3):209-49.

205. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov.* 2022;12(1):31-46.

206. Hanahan D, and Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* 2011;144(5):646-74.

207. Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, Kitui SK, and Manyazewal T. New approaches and procedures for cancer treatment: Current perspectives. *SAGE Open Med.* 2021;9(20503121211034366.

208. Jin S, Sun Y, Liang X, Gu X, Ning J, Xu Y, Chen S, and Pan L. Emerging new therapeutic antibody derivatives for cancer treatment. *Signal Transduct Target Ther.* 2022;7(1):39.
209. Hafeez U, Gan HK, and Scott AM. Monoclonal antibodies as immunomodulatory therapy against cancer and autoimmune diseases. *Curr Opin Pharmacol.* 2018;41(114-21.
210. Wang Y, Zhang H, Liu C, Wang Z, Wu W, Zhang N, Zhang L, Hu J, Luo P, Zhang J, et al. Immune checkpoint modulators in cancer immunotherapy: recent advances and emerging concepts. *J Hematol Oncol.* 2022;15(1):111.
211. Gonzalez H, Hagerling C, and Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev.* 2018;32(19-20):1267-84.
212. Hiam-Galvez KJ, Allen BM, and Spitzer MH. Systemic immunity in cancer. *Nat Rev Cancer.* 2021;21(6):345-59.
213. Hanahan D, and Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell.* 2012;21(3):309-22.
214. Jaillon S, Ponzetta A, Di Mitri D, Santoni A, Bonecchi R, and Mantovani A. Neutrophil diversity and plasticity in tumour progression and therapy. *Nat Rev Cancer.* 2020;20(9):485-503.
215. Liu J, Geng X, Hou J, and Wu G. New insights into M1/M2 macrophages: key modulators in cancer progression. *Cancer Cell Int.* 2021;21(1):389.
216. Olingy CE, Dinh HQ, and Hedrick CC. Monocyte heterogeneity and functions in cancer. *J Leukoc Biol.* 2019;106(2):309-22.
217. Raskov H, Orhan A, Christensen JP, and Gogenur I. Cytotoxic CD8(+) T cells in cancer and cancer immunotherapy. *Br J Cancer.* 2021;124(2):359-67.
218. Ben Khelil M, Godet Y, Abdeljaoued S, Borg C, Adotevi O, and Loyon R. Harnessing Antitumor CD4(+) T Cells for Cancer Immunotherapy. *Cancers (Basel).* 2022;14(1).
219. Michaud D, Steward CR, Mirlekar B, and Pylayeva-Gupta Y. Regulatory B cells in cancer. *Immunol Rev.* 2021;299(1):74-92.
220. Pitt JM, Andre F, Amigorena S, Soria JC, Eggermont A, Kroemer G, and Zitvogel L. Dendritic cell-derived exosomes for cancer therapy. *J Clin Invest.* 2016;126(4):1224-32.
221. Ostrand-Rosenberg S, and Fenselau C. Myeloid-Derived Suppressor Cells: Immune-Suppressive Cells That Impair Antitumor Immunity and Are Sculpted by Their Environment. *J Immunol.* 2018;200(2):422-31.

222. Shang J, Zha H, and Sun Y. Phenotypes, Functions, and Clinical Relevance of Regulatory B Cells in Cancer. *Front Immunol.* 2020;11(582657.
223. Syed Khaja AS, Toor SM, El Salhat H, Faour I, Ul Haq N, Ali BR, and Elkord E. Preferential accumulation of regulatory T cells with highly immunosuppressive characteristics in breast tumor microenvironment. *Oncotarget.* 2017;8(20):33159-71.
224. Saleh R, and Elkord E. FoxP3(+) T regulatory cells in cancer: Prognostic biomarkers and therapeutic targets. *Cancer Lett.* 2020;490(174-85.
225. Gonzalez-Navajas JM, Fan DD, Yang S, Yang FM, Lozano-Ruiz B, Shen L, and Lee J. The Impact of Tregs on the Anticancer Immunity and the Efficacy of Immune Checkpoint Inhibitor Therapies. *Front Immunol.* 2021;12(625783.
226. Watson MJ, Vignali PDA, Mullett SJ, Overacre-Delgoffe AE, Peralta RM, Grebinoski S, Menk AV, Rittenhouse NL, DePeaux K, Whetstone RD, et al. Metabolic support of tumour-infiltrating regulatory T cells by lactic acid. *Nature.* 2021;591(7851):645-51.
227. Aristin Revilla S, Kranenburg O, and Coffer PJ. Colorectal Cancer-Infiltrating Regulatory T Cells: Functional Heterogeneity, Metabolic Adaptation, and Therapeutic Targeting. *Front Immunol.* 2022;13(903564.
228. Pickup M, Novitskiy S, and Moses HL. The roles of TGFbeta in the tumour microenvironment. *Nat Rev Cancer.* 2013;13(11):788-99.
229. Vignali DA, Collison LW, and Workman CJ. How regulatory T cells work. *Nat Rev Immunol.* 2008;8(7):523-32.
230. Schmidt A, Oberle N, and Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol.* 2012;3(51.
231. Kannagi R, Yin J, Miyazaki K, and Izawa M. Current relevance of incomplete synthesis and neo-synthesis for cancer-associated alteration of carbohydrate determinants--Hakomori's concepts revisited. *Biochim Biophys Acta.* 2008;1780(3):525-31.
232. Houvast RD, Vankemmelbeke M, Durrant LG, Wuhrer M, Baart VM, Kuppen PJK, de Geus-Oei LF, Vahrmeijer AL, and Sier CFM. Targeting Glycans and Heavily Glycosylated Proteins for Tumor Imaging. *Cancers (Basel).* 2020;12(12).
233. Rodrigues Mantuano N, Natoli M, Zippelius A, and Laubli H. Tumor-associated carbohydrates and immunomodulatory lectins as targets for cancer immunotherapy. *J Immunother Cancer.* 2020;8(2).
234. Ahrens TD, Bang-Christensen SR, Jorgensen AM, Loppke C, Spliid CB, Sand NT, Clausen TM, Salanti A, and Agerbaek MO. The Role of Proteoglycans in

Cancer Metastasis and Circulating Tumor Cell Analysis. *Front Cell Dev Biol.* 2020;8(749.

235. Lumibao JC, Tremblay JR, Hsu J, and Engle DD. Altered glycosylation in pancreatic cancer and beyond. *J Exp Med.* 2022;219(6).
236. Navarro P, Martinez-Bosch N, Blidner AG, and Rabinovich GA. Impact of Galectins in Resistance to Anticancer Therapies. *Clin Cancer Res.* 2020;26(23):6086-101.
237. Boligan KF, Mesa C, Fernandez LE, and von Gunten S. Cancer intelligence acquired (CIA): tumor glycosylation and sialylation codes dismantling antitumor defense. *Cell Mol Life Sci.* 2015;72(7):1231-48.
238. Mereiter S, Balmana M, Campos D, Gomes J, and Reis CA. Glycosylation in the Era of Cancer-Targeted Therapy: Where Are We Heading? *Cancer Cell.* 2019;36(1):6-16.
239. RodrIguez E, Schetters STT, and van Kooyk Y. The tumour glyco-code as a novel immune checkpoint for immunotherapy. *Nat Rev Immunol.* 2018;18(3):204-11.
240. Chung JS, Tamura K, Cruz PD, Jr., and Ariizumi K. DC-HIL-expressing myelomonocytic cells are critical promoters of melanoma growth. *J Invest Dermatol.* 2014;134(11):2784-94.
241. Cherfils-Vicini J, Iltis C, Cervera L, Pisano S, Croce O, Sadouni N, Gyorffy B, Collet R, Renault VM, Rey-Millet M, et al. Cancer cells induce immune escape via glycocalyx changes controlled by the telomeric protein TRF2. *EMBO J.* 2019;38(11).
242. Kufe DW. Mucins in cancer: function, prognosis and therapy. *Nat Rev Cancer.* 2009;9(12):874-85.
243. Silsirivanit A. Glycosylation markers in cancer. *Adv Clin Chem.* 2019;89(189-213.
244. Gil J, Alvarez R, Vinuela JE, Ruiz de Morales JG, Bustos A, De la Concha EG, and Subiza JL. Inhibition of in vivo tumor growth by a monoclonal IgM antibody recognizing tumor cell surface carbohydrates. *Cancer Res.* 1990;50(22):7301-6.
245. Vinuela JE, Rodriguez R, Gil J, Coll J, De la Concha EG, and Subiza JL. Antigen shedding vs. development of natural suppressor cells as mechanism of tumor escape in mice bearing Ehrlich tumor. *Int J Cancer.* 1991;47(1):86-91.
246. Carry PJ, Prescott DM, and Ogilvie GK. Resistance to Ehrlich ascites tumor in a strain of dystrophic mice. *Cancer Res.* 1979;39(6 Pt 1):2139-40.
247. Chen L, and Watkins JF. Evidence against the presence of H2 histocompatibility antigens in Ehrlich ascites tumour cells. *Nature.* 1970;225(5234):734-5.
248. Subiza JL, Gil J, Rodriguez R, Ruiz de Morales JG, Vinuela JE, and de la Concha EG. Tumor cytostasis mediated by a monoclonal IgM antibody promoting

adhesion between macrophages and tumor cells. Evidence for a lectin-like behavior. *J Immunol.* 1992;148(8):2636-42.

249. Subiza JL, Coll J, Alvarez R, Valdivieso M, and de la Concha EG. IgM response and resistance to ascites tumor growth. *Cancer Immunol Immunother.* 1987;25(2):87-92.
250. Medina M, Velez D, Asenjo JA, Egea G, Real FX, Gil J, and Subiza JL. Human colon adenocarcinomas express a MUC1-associated novel carbohydrate epitope on core mucin glycans defined by a monoclonal antibody (A10) raised against murine Ehrlich tumor cells. *Cancer Res.* 1999;59(5):1061-70.
251. Dominguez-Andres J, Arts RJW, Bekkering S, Bahrar H, Blok BA, de Bree LCJ, Bruno M, Bulut O, Debisarun PA, Dijkstra H, et al. In vitro induction of trained immunity in adherent human monocytes. *STAR Protoc.* 2021;2(1):100365.
252. Grosche L, Knippertz I, Konig C, Royzman D, Wild AB, Zinser E, Sticht H, Muller YA, Steinkasserer A, and Lechmann M. The CD83 Molecule - An Important Immune Checkpoint. *Front Immunol.* 2020;11(721.
253. Netea MG, Joosten LA, van der Meer JW, Kullberg BJ, and van de Veerdonk FL. Immune defence against Candida fungal infections. *Nat Rev Immunol.* 2015;15(10):630-42.
254. Hernandez-Santos N, and Gaffen SL. Th17 cells in immunity to Candida albicans. *Cell Host Microbe.* 2012;11(5):425-35.
255. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Jr., et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity.* 2008;28(4):546-58.
256. Izcue A, Coombes JL, and Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev.* 2006;212(256-71.
257. Li J, Tan J, Martino MM, and Lui KO. Regulatory T-Cells: Potential Regulator of Tissue Repair and Regeneration. *Front Immunol.* 2018;9(585.
258. Campbell C, and Rudensky A. Roles of Regulatory T Cells in Tissue Pathophysiology and Metabolism. *Cell Metab.* 2020;31(1):18-25.
259. Netea MG, Brown GD, Kullberg BJ, and Gow NA. An integrated model of the recognition of Candida albicans by the innate immune system. *Nat Rev Microbiol.* 2008;6(1):67-78.
260. Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, Gow NA, Williams DL, van der Meer JW, and Netea MG. Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. *Clin Vaccine Immunol.* 2014;21(4):534-45.

261. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, Volke M, Glasner J, Warnecke C, Wiesener MS, et al. Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. *J Immunol.* 2008;180(7):4697-705.

262. Pantel A, Teixeira A, Haddad E, Wood EG, Steinman RM, and Longhi MP. Direct type I IFN but not MDA5/TLR3 activation of dendritic cells is required for maturation and metabolic shift to glycolysis after poly IC stimulation. *PLoS Biol.* 2014;12(1):e1001759.

263. Dominguez-Andres J, and Netea MG. Long-term reprogramming of the innate immune system. *J Leukoc Biol.* 2019;105(2):329-38.

264. Novakovic B, Habibi E, Wang SY, Arts RJW, Davar R, Megchelenbrink W, Kim B, Kuznetsova T, Kox M, Zwaag J, et al. beta-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. *Cell.* 2016;167(5):1354-68 e14.

265. Arts RJ, Blok BA, van Crevel R, Joosten LA, Aaby P, Benn CS, and Netea MG. Vitamin A induces inhibitory histone methylation modifications and down-regulates trained immunity in human monocytes. *J Leukoc Biol.* 2015;98(1):129-36.

266. Angelidou A, Diray-Arce J, Conti MG, Netea MG, Blok BA, Liu M, Sanchez-Schmitz G, Ozonoff A, van Haren SD, and Levy O. Human Newborn Monocytes Demonstrate Distinct BCG-Induced Primary and Trained Innate Cytokine Production and Metabolic Activation In Vitro. *Front Immunol.* 2021;12(674334.

267. Smeekens SP, Gresnigt MS, Becker KL, Cheng SC, Netea SA, Jacobs L, Jansen T, van de Veerdonk FL, Williams DL, Joosten LA, et al. An anti-inflammatory property of Candida albicans beta-glucan: Induction of high levels of interleukin-1 receptor antagonist via a Dectin-1/CR3 independent mechanism. *Cytokine.* 2015;71(2):215-22.

268. Gresnigt MS, Bozza S, Becker KL, Joosten LA, Abdollahi-Roodsaz S, van der Berg WB, Dinarello CA, Netea MG, Fontaine T, De Luca A, et al. A polysaccharide virulence factor from Aspergillus fumigatus elicits anti-inflammatory effects through induction of Interleukin-1 receptor antagonist. *PLoS Pathog.* 2014;10(3):e1003936.

269. de Bree LCJ, Koeken V, Joosten LAB, Aaby P, Benn CS, van Crevel R, and Netea MG. Non-specific effects of vaccines: Current evidence and potential implications. *Semin Immunol.* 2018;39(35-43.

270. Subiza JL, Vinuela JE, Rodriguez R, Gil J, Figueredo MA, and De La Concha EG. Development of splenic natural suppressor (NS) cells in Ehrlich tumor-bearing mice. *Int J Cancer.* 1989;44(2):307-14.

271. Ruiz de Morales J, Velez D, and Subiza JL. Ehrlich tumor stimulates extramedullar hematopoiesis in mice without secreting identifiable colony-stimulating factors and without engagement of host T cells. *Exp Hematol.* 1999;27(12):1757-67.
272. Oh SA, Wu DC, Cheung J, Navarro A, Xiong H, Cubas R, Totpal K, Chiu H, Wu Y, Comps-Agrar L, et al. PD-L1 expression by dendritic cells is a key regulator of T-cell immunity in cancer. *Nat Cancer.* 2020;1(7):681-91.
273. Mayoux M, Roller A, Pulko V, Sammicheli S, Chen S, Sum E, Jost C, Fransen MF, Buser RB, Kowanetz M, et al. Dendritic cells dictate responses to PD-L1 blockade cancer immunotherapy. *Sci Transl Med.* 2020;12(534).
274. Bakdash G, Vogelpoel LT, van Capel TM, Kapsenberg ML, and de Jong EC. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. *Mucosal Immunol.* 2015;8(2):265-78.
275. Sobah ML, Liongue C, and Ward AC. SOCS Proteins in Immunity, Inflammatory Diseases, and Immune-Related Cancer. *Front Med (Lausanne).* 2021;8(727987.
276. Feichtinger RG, and Lang R. Targeting L-Lactate Metabolism to Overcome Resistance to Immune Therapy of Melanoma and Other Tumor Entities. *J Oncol.* 2019;2019(2084195.
277. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, Mackensen A, and Kreutz M. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood.* 2006;107(5):2013-21.
278. Raychaudhuri D, Bhattacharya R, Sinha BP, Liu CSC, Ghosh AR, Rahaman O, Bandopadhyay P, Sarif J, D'Rozario R, Paul S, et al. Lactate Induces Pro-tumor Reprogramming in Intratumoral Plasmacytoid Dendritic Cells. *Front Immunol.* 2019;10(1878.
279. Collins LE, and Troeberg L. Heparan sulfate as a regulator of inflammation and immunity. *J Leukoc Biol.* 2019;105(1):81-92.
280. Marques C, Reis CA, Vives RR, and Magalhaes A. Heparan Sulfate Biosynthesis and Sulfation Profiles as Modulators of Cancer Signalling and Progression. *Front Oncol.* 2021;11(778752.
281. Karamanos NK, Piperigkou Z, Theocharis AD, Watanabe H, Franchi M, Baud S, Brezillon S, Gotte M, Passi A, Vigetti D, et al. Proteoglycan Chemical Diversity Drives Multifunctional Cell Regulation and Therapeutics. *Chem Rev.* 2018;118(18):9152-232.
282. Hassan N, Greve B, Espinoza-Sanchez NA, and Gotte M. Cell-surface heparan sulfate proteoglycans as multifunctional integrators of signaling in cancer. *Cell Signal.* 2021;77(109822.

283. De Pasquale V, and Pavone LM. Heparan Sulfate Proteoglycan Signaling in Tumor Microenvironment. *Int J Mol Sci.* 2020;21(18).

284. Farrugia BL, Lord MS, Melrose J, and Whitelock JM. The Role of Heparan Sulfate in Inflammation, and the Development of Biomimetics as Anti-Inflammatory Strategies. *J Histochem Cytochem.* 2018;66(4):321-36.

285. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol.* 2004;4(12):941-52.

286. Tran Janco JM, Lamichhane P, Karyampudi L, and Knutson KL. Tumor-infiltrating dendritic cells in cancer pathogenesis. *J Immunol.* 2015;194(7):2985-91.

287. Veglia F, and Gabrilovich DI. Dendritic cells in cancer: the role revisited. *Curr Opin Immunol.* 2017;45(43-51.

288. Laoui D, Keirsse J, Morias Y, Van Overmeire E, Geeraerts X, Elkrim Y, Kiss M, Bolli E, Lahmar Q, Sichien D, et al. The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nat Commun.* 2016;7(13720.

289. Ohradanova-Repic A, Machacek C, Fischer MB, and Stockinger H. Differentiation of human monocytes and derived subsets of macrophages and dendritic cells by the HLDA10 monoclonal antibody panel. *Clin Transl Immunology.* 2016;5(1):e55.

290. Krakow S, Crescimone ML, Bartels C, Wiegering V, Eyrich M, Schlegel PG, and Wolfl M. Re-expression of CD14 in Response to a Combined IL-10/TLR Stimulus Defines Monocyte-Derived Cells With an Immunoregulatory Phenotype. *Front Immunol.* 2019;10(1484.

291. Unger WW, Laban S, Kleijwegt FS, van der Slik AR, and Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol.* 2009;39(11):3147-59.

292. Ramos RN, Chin LS, Dos Santos AP, Bergami-Santos PC, Laginha F, and Barbuto JA. Monocyte-derived dendritic cells from breast cancer patients are biased to induce CD4+CD25+Foxp3+ regulatory T cells. *J Leukoc Biol.* 2012;92(3):673-82.

293. Surowka J, Wertel I, Okla K, Bednarek W, Tarkowski R, and Kotarski J. Influence of ovarian cancer type I and type II microenvironment on the phenotype and function of monocyte-derived dendritic cells. *Clin Transl Oncol.* 2017;19(12):1489-97.

294. Watson MJ, and Delgoffe GM. Fighting in a wasteland: deleterious metabolites and antitumor immunity. *J Clin Invest.* 2022;132(2).

APPENDIX

List of publications:

- **Martín-Cruz L**, Sevilla-Ortega C, Benito-Villalvilla C, Diez-Rivero CM, Sánchez-Ramón S, Subiza JL, Palomares O. A Combination of Polybacterial MV140 and Candida albicans V132 as a Potential Novel Trained Immunity-Based Vaccine for Genitourinary Tract Infections. Front Immunol. 2021;21;11:612269. doi: 10.3389/fimmu.2020.612269. (Impact factor: 8.786; Q1 35/162 in 'Immunology').

- **Martín-Cruz L**, Angelina A, Baydemir I, Bulut Ö, Subiza JL, Netea MG, Domínguez-Andrés J[#], Palomares O[#]. *Candida albicans* V132 induces trained immunity and enhances the responses triggered by the polybacterial vaccine MV140 for genitourinary tract infections. Front Immunol. 2022;13:1066383. doi: 10.3389/fimmu.2022.1066383. (Impact factor: 8.786; Q1 35/162 in 'Immunology'). [#]These authors share last authorship.

- **Martín-Cruz L***, Sevilla-Ortega C*, Angelina A, Domínguez-Andrés J, Netea MG, Subiza JL, Palomares O. From trained immunity in allergy to trained immunity-based allergen vaccines. Clin Exp Allergy. 2022. doi: 10.1111/cea.14261. (Impact factor: 5.401; Q2 13/28 in 'Allergy'). *These authors equally contributed to this work.

- Angelina A*, **Martín-Cruz L***, de la Rocha-Muñoz A, Lavín-Plaza B, Palomares O. C-type Lectin Receptor Mediated Modulation of T2 Immune Responses to Allergens. Curr Allergy Asthma Rep. 2022. Accepted. (Impact factor: 4.919; Q2 78/162 in 'Immunology'). *These authors equally contributed to this work.

frontiers in Immunology

ORIGINAL RESEARCH
published: 21 January 2021
doi: 10.3389/fimmu.2020.612269

A Combination of Polybacterial MV140 and *Candida albicans* V132 as a Potential Novel Trained Immunity-Based Vaccine for Genitourinary Tract Infections

Leticia Martin-Cruz[1], *Carmen Sevilla-Ortega*[1], *Cristina Benito-Villalvilla*[1], *Carmen M. Diez-Rivero*[2], *Silvia Sanchez-Ramón*[3,4], *José Luis Subiza*[2] *and Oscar Palomares*[1]*

[1] Department of Biochemistry and Molecular Biology, School of Chemistry, Complutense University, Madrid, Spain, [2] Inmunotek, Alcalá de Henares, Madrid, Spain, [3] Department of Clinical Immunology and IdISSC, Hospital Clínico San Carlos, Madrid, Spain, [4] Department of Immunology, ENT and Ophthalmology, School of Medicine, Complutense University, Madrid, Spain

OPEN ACCESS

Edited by:
África González-Fernández, University of Vigo, Spain

Reviewed by:
Javier Carbone, Gregorio Marañón Hospital, Spain
Salvador Iborra, Universidad Complutense de Madrid, Spain

***Correspondence:**
Oscar Palomares
oscar.palomares@quim.ucm.es

Specialty section:
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

Received: 30 September 2020
Accepted: 03 December 2020
Published: 21 January 2021

Citation:
Martin-Cruz L, Sevilla-Ortega C, Benito-Villalvilla C, Diez-Rivero CM, Sanchez-Ramón S, Subiza JL and Palomares O (2021) A Combination of Polybacterial MV140 and Candida albicans V132 as a Potential Novel Trained Immunity-Based Vaccine for Genitourinary Tract Infections. Front. Immunol. 11:612269. doi: 10.3389/fimmu.2020.612269

Recurrent urinary tract infections (RUTIs) and recurrent vulvovaginal candidiasis (RVVCs) represent major healthcare problems with high socio-economic impact worldwide. Antibiotic and antifungal prophylaxis remain the gold standard treatments for RUTIs and RVVCs, contributing to the massive rise of antimicrobial resistance, microbiota alterations and co-infections. Therefore, the development of novel vaccine strategies for these infections are sorely needed. The sublingual heat-inactivated polyvalent bacterial vaccine MV140 shows clinical efficacy for the prevention of RUTIs and promotes Th1/Th17 and IL-10 immune responses. V132 is a sublingual preparation of heat-inactivated *Candida albicans* developed against RVVCs. A vaccine formulation combining both MV140 and V132 might well represent a suitable approach for concomitant genitourinary tract infections (GUTIs), but detailed mechanistic preclinical studies are still needed. Herein, we showed that the combination of MV140 and V132 imprints human dendritic cells (DCs) with the capacity to polarize potent IFN-γ– and IL-17A–producing T cells and FOXP3$^+$ regulatory T (Treg) cells. MV140/V132 activates mitogen-activated protein kinases (MAPK)-, nuclear factor-κB (NF-κB)- and mammalian target of rapamycin (mTOR)-mediated signaling pathways in human DCs. MV140/V132 also promotes metabolic and epigenetic reprogramming in human DCs, which are key molecular mechanisms involved in the induction of innate trained immunity. Splenocytes from mice sublingually immunized with MV140/V132 display enhanced proliferative responses of CD4$^+$ T cells not only upon *in vitro* stimulation with the related antigens contained in the vaccine formulation but also upon stimulation with phytohaemagglutinin. Additionally, *in vivo* sublingual immunization with MV140/V132 induces the generation of IgG and IgA antibodies against all the components contained in the vaccine formulation. We uncover immunological mechanisms underlying the potential mode of action of a

combination of MV140 and V132 as a novel promising trained immunity-based vaccine (TIbV) for GUTIs.

Keywords: dendritic cells, recurrent urinary tract infections (RUTIs), recurrent vulvovaginal candidiasis (RVVCs), trained immunity-based vaccines (TIbVs), *candida albicans* V132, polybacterial preparation MV140

INTRODUCTION

Bacterial infections represent a major health-care problem and among them, urinary tract infections (UTIs) are one of the most common with a high incidence in women (1–4). UTIs are considered recurrent (RUTIs) with two or more symptomatic infections in less than six months or more than three per year (2, 5). Antibiotic treatment is the current therapy used for RUTIs, but its long-term use increases the risk to develop antibiotic resistance (3, 6). The overuse of antibiotics is also associated with dysregulation of normal vagina and gastrointestinal microbiota, which favor pathogen invasion and subsequent bacterial and fungal infection (1, 3, 7). Due to microbiota alterations, opportunistic pathogens such as *Candida albicans* colonize the vaginal tract generating vulvovaginal candidiasis (VVCs) (8–12). More than 70% of women worldwide suffer from VVCs (with maximum incidence between 20 and 40 years old) and around 5% experience recurrent infections (RVVCs), defined as four or more episodes per year (13–16). RVVCs require antifungal therapy to avoid recurrence and its overuse involves antimycotic resistances (17–19). Both RUTIs and RVVCs markedly diminish quality of life in women, with a negative impact at work and social life (20, 21). Therefore, there is an urgent need to develop novel strategies for concomitant and recurrent genitourinary tract infections (GUTIs), including RUTIs and RVVCs (1, 4, 16–18, 22).

Mucosal bacterial or fungal vaccines have been proposed as a new alternative approach to antibiotics and antifungals, respectively (23–25). These preparations containing soluble antigens or inactivated whole pathogens might well induce both specific immune responses and also non-specific immunomodulation. Sublingual administration of mucosal vaccines based on whole inactivated components has been recently reported as a suitable strategy to prevent and treat recurrent infections (24, 26–28). These preparations proposed as trained immunity-based vaccines (TIbVs) are able to induce long-lasting changes in innate immune cells, including dendritic cells (DCs), that enhance responses and protection against both the pathogens included in the vaccine formulation but also against unrelated pathogens by mechanisms involving immunological, metabolic and epigenetic reprogramming (23, 29–32). DCs survey the mucosal environment using innate pattern recognition receptors (PRRs), and are responsible for linking innate and adaptive immune responses (33, 34). Activation of DCs is linked to metabolic changes coupling the energetic needs with the functional activity (35, 36). Activation of DCs with microbial components promotes Warburg effect, a shift from oxidative phosphorylation to glycolysis and lactic fermentation under normal oxygen conditions (35, 37). This metabolic rewiring in DCs is driven at the molecular level by different mechanisms including activation of the axis protein kinase B (Akt)/mammalian target of rapamycin (mTOR)/hypoxia inducible factor-1α (HIF-1α) (35, 36).

MV140 (Uromune) is a polyvalent bacterial preparation based on whole heat-inactivated components used to prevent RUTIs (38). This sublingual vaccine is composed of bacteria causing the majority of RUTIs in Europe: 75% Gram-negative bacteria (25% *Escherichia coli*, 25% *Proteus vulgaris* and 25% *Klebsiella pneumoniae*) and 25% Gram-positive bacteria (*Enterococcus faecalis*) (26). Clinical data showed that MV140 significantly reduced the infection rates in patients suffering from RUTIs (39–43). MV140 acts directly on human DCs promoting maturation and activation through mechanisms depending on Syk- and MyD88-mediated signaling pathways, supporting the involvement of C-type lectin receptors (CLRs) and toll-like receptors (TLRs). MV140-induced downstream activation of NF-κB and p38 in DCs contribute to Th1/Th17 and IL-10 polarization whereas JNK and ERK are involved in Th1 and IL-10 immune responses, respectively (26). V132 is a preparation of heat-inactivated *C. albicans* that has been developed as a possible treatment of RVVCs. Given the tight relationship between RUTIs and RVVCs, a polymicrobial formulation, *via* combination of MV140 and V132, might well represent a suitable vaccine for both diseases.

In this study, we demonstrated that the combination of MV140 and V132 imprints human DCs with the capacity to generate potent IFN-γ– and IL-17A–producing T cells and FOXP3$^+$ regulatory T (Treg) cells. Mechanistically, we showed that MV140/V132 activates inflammatory and mTOR pathways as well as metabolic and epigenetic reprogramming in human DCs. *In vivo*, sublingual immunization of BALB/c mice with MV140/V132 enhances the proliferation of splenic CD4$^+$ T cells upon *in vitro* stimulation with the antigens contained in the formulation and with phytohaemagglutinin (PHA). Sublingual immunization with MV140/V132 also induces the generation of specific IgG and IgA antibodies against all the components contained in the formulation. Overall, we show for the first time molecular mechanisms that

Abbreviations: Akt, protein kinase B; APC, allophycocyanin; CLRs, C-type lectin receptors; COXII, cyclooxygenase II; DCs, dendritic cells; FITC, fluorescein isothiocyanate; FTU, Formazan Turbidity Units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GUTIs, genitourinary tract infections; HIF-1α, hypoxia inducible factor-1α; hmoDCs, human monocyte-derived DCs; MAPK, mitogen-activated protein kinases; MTA, 5′-deoxy-5′-(methylthio)adenosine; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cells; PBP, polyvalent bacterial preparation; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; PHA, phytohaemagglutinin; RUTIs, recurrent urinary tract infections; RVVCs, recurrent vulvovaginal candidiasis; TIbV, trained immunity-based vaccines; TLRs, toll-like receptors; Treg, regulatory T; UTIs, urinary tract infections; VVCs, vulvovaginal candidiasis.

might be involved in the mode of action of MV140/V132 as a novel promising trained immunity-based vaccine (TIbV) for GUTIs.

MATERIALS AND METHODS

Media and Reagents

RPMI 1640 (Lonza) supplemented with 10% fetal bovine serum, 100 μg/ml normocin, 50 μg/ml penicillin-streptomycin, 1% non-essential amino acids, 1% MEM-vitamins and 1 mM sodium pyruvate. MV140 (Uromune) composed of whole heat-inactivated bacteria (25% *E. coli*, 25% *P. vulgaris*, 25% *K. pneumoniae* and 25% *E. faecalis*), V132 composed of whole heat-inactivated *C. albicans*, MV140/V132 (combination of MV140 and V132) and control excipients (negative control, containing all excipients without bacteria and yeast) were provided by Inmunotek S.L. Inhibitors for histone methyltransferase 5′-deoxy-5′-(methylthio) adenosine (MTA) and histone demethylase (Pargyline) (Sigma-Aldrich) were used for the inhibition experiments.

Generation of Human Monocyte-Derived Dendritic Cells and Purification of Naïve CD4$^+$ T Cells

Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were obtained by using Ficoll density gradient centrifugation (800*g*, 20 min). Monocytes were isolated from total PBMC using anti-CD14 microbeads and cultured for 6 days with RPMI medium containing 100 ng/ml of IL-4 and GM-CSF (PeproTeck) to generate immature human monocyte-derived DCs (hmoDCs). The purity and phenotype of monocytes and generated immature hmoDCs were analyzed by flow cytometry with lineage-specific markers. Peripheral blood naive CD4$^+$ T cells were isolated using the "Naive CD4$^+$ T Cell Isolation Kit" (Miltenyi Biotec), according to manufacturer's protocol.

Cell Cultures

Immature hmoDCs (10^6 cells per ml) were stimulated with control excipients (containing all excipients except the microbial components), V132 (3 FTU, Formazan Turbidity Units, per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml) for 18 h. Subsequently, cells were collected and centrifuged. Cell pellets were used to analyze their phenotype by flow cytometry and cell-free supernatants were used to quantify IL-6, TNF-α, IL-23, IL-1β, IL-10, and TGF-β1 by ELISA. For inhibition experiments, hmoDCs were preincubated for 1 h with MTA (0.5 mM) or Pargyline (3 μM) (or their vehicle control, DMSO) prior to activation. Then, the cells were stimulated with stimuli for 18 h in the presence of the corresponding inhibitors to quantify TNF-α, IL-23, and IL-10 by ELISA. Cell viability was analyzed in all the cases by trypan blue exclusion with a light microscope.

Flow Cytometry

The following anti-human monoclonal antibodies (mAbs) were used for flow cytometry: fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR; phycoerythrin (PE)-conjugated anti-CD86; and allophycocyanin (APC)-conjugated anti-CD83 (Miltenyi Biotec). APC-conjugated anti-CD3; and Alexa Fluor 488-conjugated anti-IFN-γ (BD Pharmigen). PE-conjugated anti-IL-10; Alexa Fluor 488-conjugated anti-IL-17A; peridinin-chlorophyll-protein (PerCP)-conjugated anti-CD4; Alexa Fluor 488-conjugated anti-FOXP3; PE-conjugated anti-CD127; and APC-conjugated anti-CD25 (Biolegend). Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 min at room temperature in the darkness. For analysis of FOXP3 expression in human T cells primed with hmoDCs, cells were first subjected to surface staining with anti-human CD127-PE, CD4-PerCP, and CD25-APC antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488, according to manufacturer's recommendations. For each staining, the corresponding isotype controls (IgG2A-FITC, IgG1-Alexa Fluor 488, IgG1-PE, IgG2A-PerCP, or IgG1-APC) were also assayed. Flow cytometry analysis was performed in a FACSCalibur in the Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

Cytokine Quantification

The cytokine production of IL-6, TNF-α, IL-1β, IL-10, IFN-γ, IL-5 (BD Biosciences), IL-23 (Invitrogen), IL-17A (Mabtech), and TGF-β1 (R&D System) was quantified by sandwich ELISA in cell-free supernatants following the manufacturer's instructions.

Co-Culture Experiments

HmoDCs were co-cultured with purified allogeneic naïve CD4$^+$ T cells (1:5 DC/T cell) for 3 days in the presence of control excipients, V132 (3 FTU per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml). IFN-γ, IL-5, IL-17A, and IL-10 were quantified in cell-free supernatants by ELISA. For proliferation assays, purified allogeneic naive CD4$^+$ T cells were labeled with CFSE (Molecular Probes) prior to co-culture with immature hmoDCs. Proliferation was assessed by measuring CFSE dilution on labeled CD4$^+$ T cells by flow cytometry. For intracellular staining, the primed CD4$^+$ T cells were washed and restimulated with 25 ng/ml of PMA plus 1 μg/ml of ionomycin for 6 h and 10 μg/ml of Brefeldin A for the last 4 h. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. The cells were stained with the combination of fluorochrome-labeled mAbs to IFN-γ, IL-10, and IL-17A.

Western Blot Analysis

HmoDCs (10^6 cells per ml) were treated with control excipients, V132 (3 FTU per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml). After 30 min at 37°C, cells were harvested and lysed with RIPA buffer (ThermoFisher scientific) in presence of Protease/Phosphatase Inhibitor Cocktail (Cell Signaling) for 30 min at 4°C vortexing every 10 min. Lysates were clarified by centrifugation at 10,000*g* for 15 min at 4°C. Protein quantification was performed with Micro BCA Protein Assay Kit (Pierce) and samples with equal amounts of total protein were resolved in 10% SDS-

polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred to nitrocellulose membrane (BioRad). The membrane was incubated with the primary antibodies: phospho-ERK1/2 (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-IKKα (Ser176)/IKKβ (Ser177), or phospho-Akt (Ser473) (1:1000, Cell Signaling), phospho-p70 S6 Kinase (Thr389) (1:750; Cell Signaling), and β-actin (1:15000, Sigma-Aldrich); and goat anti-rabbit (1:4000, BioRad) or goat anti-mouse (1:2500, Pierce) conjugate with horseradish peroxidase as a secondary antibody. The signal was developed with Clarity Western ECL Substrate (Bio-Rad) and detected in a Fujifilm LAS-3000 developer.

Metabolic Studies

HmoDCs were stimulated with control excipients, V132 (3 FTU per ml), MV140 (10^7 bact. per ml) or MV140/V132 (MV140, 10^7 bact. per ml, and V132, 3 FTU per ml) for 18 h. Glucose concentration in cell-free supernatants was determined by using the Glucose (GO) Assay Kit (Sigma-Aldrich). The metabolic rate was derived mathematically in percentage of medium without hmoDCs (2 mg/ml). Lactate production in cell-free supernantants was determined by using the colorimetric L-Lactate Assay kit (Sigma-Aldrich). The mitochondrial membrane potential was measured using MitoTracker Red Mitochondrion-Selective Probe kit (ThermoFisher Scientific) following the manufacturer's instructions with minor modifications. Briefly, mitochondrial activation was measured using MitoTracker Red CMXROS (250 nM) for 30 min at 37°C after 18 h of treatment with the corresponding stimulus. The fluorescence of the probe was analyzed using FLUOstar OPTIMA fluorescence reader (BMG LabTech).

RNA Extraction, cDNA Synthesis, and Quantitative Real-time RT-PCR

RNA was isolated from harvested cells using an RNeasy mini kit (Qiagen) and cDNA generated using a PrimeScript RT reagent Kit (Takara) according to manufacturer's instructions. Real-time quantitative PCR was performed on cDNA using FastStart Universal SYBR Green Master (Rox) (Roche). The sequences of the used pair primers were: cyclooxygenase II (COXII) (forward, CTATCCTGCCCGCCATCATC; reverse, GGGATCGTTGACCTCGTCTG), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, CTGCACCACCAACTGC TTAGC; reverse, TCATGTTCTGGAGAGCCCCG), Hypoxia-inducible factor 1α (HIF-1α) (forward, TCGCATCTTGATAA GGCCTCT; reverse, ACAAAACCATCCAAGGCTTTCA), and Elongation Factor 1α (EF1α) (forward, CTGAACCATCCAG GCCAAAT; reverse, GCCGTGTGGCAATCCAAT). Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). Data were normalized to EF1α and displayed as arbitrary units calculated as $2^{-\Delta CT}$ values multiplied by 10^4. ΔCT was defined as the difference between the cycle threshold value for the gene of interest and EF1α. The mitochondrial/nuclear DNA content was calculated by using the formula: $2^{-\Delta CT}$ mitochondrial COXII/$2^{-\Delta CT}$ nuclear GAPDH.

Sublingual Immunization of Mice With MV140/V132 or Control Excipients: Quantification of CD4$^+$ T Cell Proliferation of *In Vitro* Stimulated Splenocytes With V132, MV140, MV140/V132, PHA, or Control Excipients, and Induced Serum-Specific Antibody Levels

BALB/c mice (6-weeks-old) were immunized four times with 20 µl of MV140/V132, MV140 alone, V132 alone (300 FTU per ml) or control (all excipients without bacteria and yeast) by sublingual administration every 7 days and killed 7 days after the last immunization. Sublingual administration was performed under anaesthesia (ketamine, 100 mg/kg and xylacine, 5 mg/kg) to ensure proper delivery. Spleens were used to prepare single cell suspensions following conventional protocols. Cells were labeled with CFSE (5 mM, Life technologies) and stimulated *in vitro* with V132 (6 FTU per ml), MV140 (6 FTU per ml), MV140/V132 (MV140, 3 FTU per ml, and V132, 3 FTU per ml), phytohaemagglutinin (PHA, 5 µg/ml, Sigma-Aldrich) or control excipients for 5 days. Proliferation of CFSE-labeled CD4$^+$ T cells was monitored by flow cytometry. Serum specific IgG, IgA, IgG1, and IgG2a for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis*, and *P. vulgaris* induced after sublingually immunization were determined by ELISA. Briefly, 96-well non-tissue culture-treated plates were pretreated with poly-L-lysine (Sigma-Aldrich) for 1 h under UV light and coated with each of the heat-inactivated whole cell bacteria or yeast (300 FTU per ml) overnight at 4°C, and, subsequently, incubated with mouse serum dilutions for 2 h at room temperature. Specific immunoglobulins were detected with biotin rat anti-mouse IgA and IgG (both from Sigma-Aldrich). Signal was developed by incubation with streptavidin-horseradish peroxidase (HRP) (Sigma-Aldrich). Peroxidase activity was revealed by the addition of o-phenylenediamine dihydrochloride (Sigma-Aldrich) and the reaction was stopped with HCl 1 N. Plates were read on an ELISA reader at 490 nm (Triturus Elisa, Grifols). Animals were maintained in Biolab S.L. under the conditions stabilized by the R.D. 53/2013 and in accordance with the international GLP normative, RD 223/83 and ISO 10993-2:2007. The tests are performed in accordance with official methods, European Pharmacopoeia, USP Pharmacopoeia, OECD Guideline, UNE EN-ISO, and specific methodologies developed for R&D.

Statistics

All the data are expressed as means ± s.e.m. of the indicated parameters. Paired or unpaired Student's t-test used for statistical analysis were performed using GraphPad Prism software, version 6.0. Significance was defined as $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

RESULTS

MV140/V132-Activated Human Dendritic Cells Display Significantly Higher Levels of CD83, Pro-Inflammatory Cytokines, and IL-10 Than Dendritic Cells Stimulated With the Individual Components

To assess the capacity of the combination of the polyvalent bacteria preparation MV140 and *C. albicans* V132 to modulate the phenotype and function of human DCs, we treated human monocyte-derived DCs (hmoDCs) from healthy donors with V132, MV140, MV140/V132 or control excipients. HmoDCs treated with V132 produced significantly higher levels of IL-6 and TNF-α, and a slight increase in the production of IL-1β and IL-10 than control excipients. IL-23 levels were no detectable in cell-free supernatants from V132-treated hmoDCs (**Figure 1A**). MV140 induced significant production of IL-6, TNF-α, IL-1β, IL-23, and IL-10 compared to control treatment, as previously described (26), and also compared to V132 (**Figure 1A**). MV140/V132-activated hmoDCs induced significantly higher levels of pro-inflammatory cytokines and IL-10 than excipients- or V132-activated hmoDCs (**Figure 1A**). Interestingly, MV140/V132 stimulation induced significantly higher levels of TNF-α, IL-23, and IL-10 than MV140 in hmoDCs (**Figure 1A**). To test whether simultaneous stimulation of hmoDCs with MV140/V132 enhance the production of TNF-α, IL-23, and IL-10 in a synergistic manner, cytokine levels induced by MV140/V132 showed in **Figure 1A** were directly compared to those resulting from the addition of the cytokine levels produced by activation with MV140 and V132 separately (**Figure 1A**). MV140/V132-activated hmoDCs induced significantly higher levels of TNF-α, IL-23, and IL-10 than the corresponding cytokine levels produced by V132- plus MV140-treated hmoDCs, supporting synergistic cooperation in the simultaneous stimulation with the combination of MV140 and V132 (**Figure 1B**). We did not detect significant differences in the levels of TGF-β1 among any of the assayed conditions (**Supplementary Figure 1A**). Although there were no significant differences in the percentage of cells expressing HLA-DR (**Figures 1C, D**), the expression levels (MFI values) were significantly increased after V132, MV140 and MV140/V132 stimulation compared to excipients-treated cells, without significant differences among them (**Figure 1E**). V132,

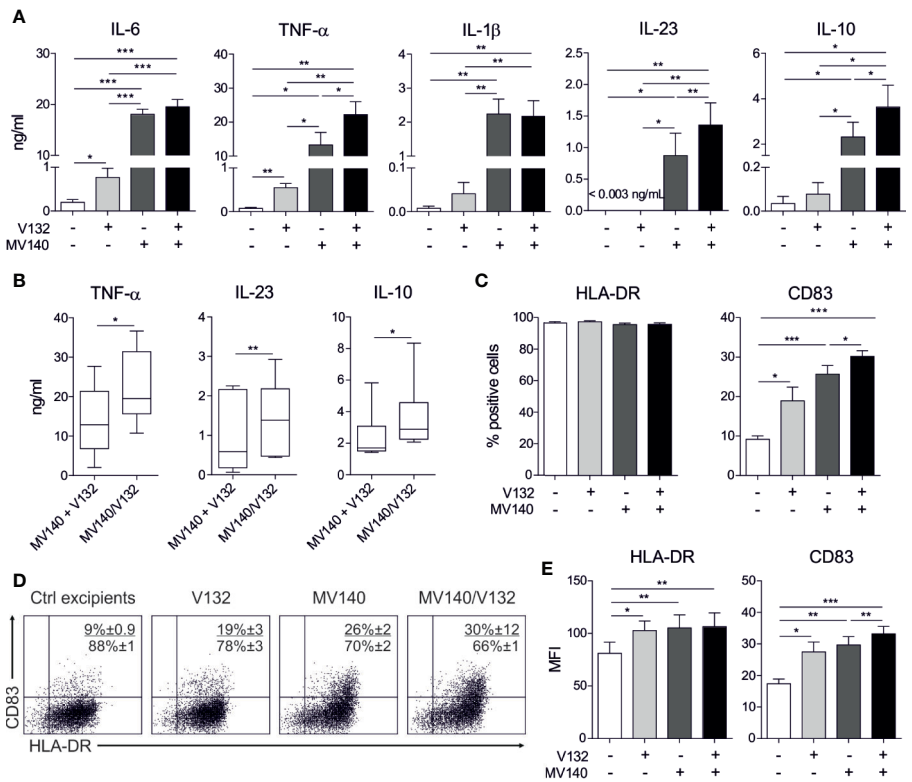

FIGURE 1 | MV140/V132 induces hmoDCs maturation and production of pro-inflammatory cytokines and IL-10. **(A)** Cytokine production after stimulation of hmoDCs with control excipients (containing all excipients except the bacteria and yeast), V132, MV140, and MV140/V132 for 18 h quantified in cell-free supernatants by ELISA. **(B)** Cytokine levels of V132-treated hmoDCs plus cytokine levels of MV140-treated hmoDCs compared with cytokine levels of MV140/V132-stimulated hmoDCs. **(C)** Percentage of CD83- and HLA-DR-positive cells after stimulation with control excipients, V132, MV140 and MV140/V132 for 18 h. **(D)** Flow cytometry representative dot plots for the expression of surface markers CD83 and HLA-DR after stimulation of hmoDCs with the indicated stimulus. **(E)** Mean Fluorescence Intensity (MFI) values for CD83 and HLA-DR. Results are mean ± s.e.m. of 6-8 **(A)**, 6 **(B)**, and 7-8 **(C, E)** independent experiments. Paired Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

MV140 and MV140/V132 significantly increased the expression of the co-stimulatory molecule CD83 in hmoDCs compared to control excipients (**Figures 1C, D**). MV140 and MV140/V132 induced higher expression of CD83 than V132 without significant differences (**Figures 1C, D**). Both the percentage of positive cells and the expression levels of CD83 were significantly higher in hmoDCs stimulated with MV140/V132 than MV140, indicating that the combination of these stimuli in a single formulation significantly enhance the maturation of hmoDCs (**Figures 1C, E**). The expression of CD86 was significantly higher in hmoDCs stimulated with V132 than the other stimuli, but no differences were observed after MV140 and MV140/V132 stimulation compared to control excipients (**Supplementary Figures 1B–D**).

MV140/V132-Activated Human Dendritic Cells Generate Potent IFN-γ- and IL-17A–Producing T Cells and FOXP3$^+$ Treg Cells

To determine the capacity of human DCs treated with the combination of MV140 and V132 to polarize CD4$^+$ T cell responses, we performed co-culture experiments. HmoDCs stimulated with MV140 and MV140/V132 induced a significantly higher percentage of proliferating allogeneic CD4$^+$ T cells than excipients-treated hmoDCs (**Figure 2A**). V132-activated hmoDCs were also able to stimulate proliferation of CD4$^+$ T cells but significant changes were not detected when comparing with excipients-treated hmoDCs (**Figure 2A**). HmoDCs activated with MV140 or MV140/V132 generated T cells producing significantly higher levels of IFN-γ, IL-17A, and IL-10 than excipients- or V132-stimulated hmoDCs (**Figure 2B**). We did not detect IL-5 production in any of the assayed conditions (**Figure 2B**). Interestingly, MV140/V132-activated hmoDCs generated T cells producing significantly higher levels of IL-17A and IL-10 than MV140-activated hmoDCs (**Figure 2B**). To assess potential synergistic effects, the levels of IL-17A and IL-10 produced by T cells generated by MV140/V132-activated hmoDCs (**Figure 2B**) were compared to those resulting from the addition of these cytokine levels in MV140- and V132-stimulated conditions separately, shown in **Figure 2B**. IL-17A and IL-10 production was significantly higher when T cells were primed by hmoDCs activated with MV140/V132 than the sum of the cytokine levels produced by T cells generated by V132- plus MV140-activated hmoDCs, thus supporting synergistic effects of MV140/V132 at the T cell level (**Figure 2C**). To verify these data at the single cell level, we performed intracellular staining experiments. The percentages of IFN-γ–, IL-10–, and IL-17A–producing CD4$^+$ T cells generated by hmoDCs treated with V132, MV140 or MV140/V132 were significantly higher than those produced by T cells generated by excipients-treated hmoDCs (**Figure 3A**). Supporting our results, MV140/V132-activated hmoDCs generated a significantly higher percentage of IL-10– and IL-17A–producing T cells than MV140-treated cells (**Figure 3A**). To investigate the capacity of activated hmoDCs to induce Treg cells, we also analyzed the generation of FOXP3$^+$ Treg cells in allogeneic co-culture experiments. V132-, MV140-, and MV140/V132-treated hmoDCs induced significantly higher percentages of CD4$^+$CD25^{high}CD127$^-$FOXP3$^+$ Treg cells than excipients-treated hmoDCs (**Figure 3B**). Remarkably, the number of FOXP3$^+$ Treg cells generated by MV140/V132-activated hmoDCs was significantly higher than those induced by hmoDCs stimulated with V132 or MV140 alone (**Figure 3B**).

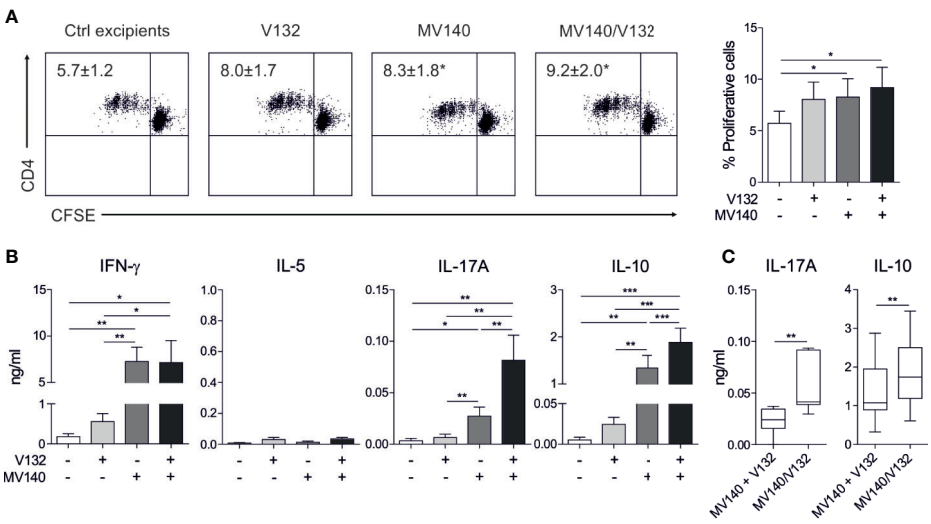

FIGURE 2 | MV140/V132-activated hmoDCs induce T cell proliferation and Th1, Th17, and IL-10 producing T cells. **(A)** Representative dot plots of proliferating CFSE-labeled allogeneic naive CD4$^+$ T cells after 3 days of co-culture with control excipients-, V132-, MV140-, and MV140/V132-stimulated hmoDCs and the graph with the frequency of proliferating cells (right side). **(B)** Cytokines produced by allogeneic naive CD4$^+$ T cells primed by hmoDCs in the presence of the indicated stimulus after 3 days quantified in cell-free supernatants by ELISA. **(C)** Levels of IL-17A and IL-10 for V132- plus MV140-stimulated co-cultures compared to MV140/V132-stimulated co-cultures. Results are mean ± s.e.m. of 5 **(A)**, and 8 to 12 **(B, C)** independent experiments. Paired Student *t* test, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

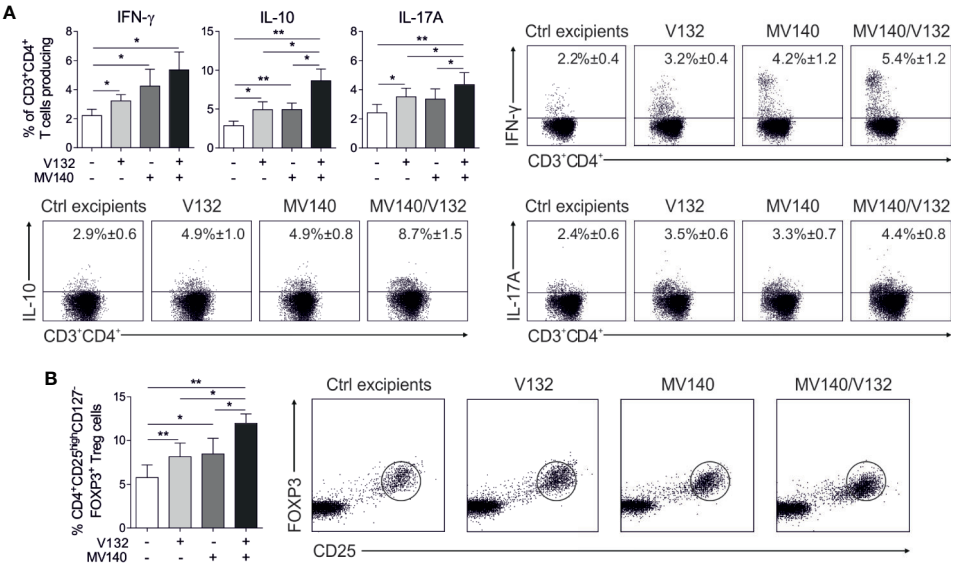

FIGURE 3 | MV140/V132-activated hmoDCs generate IFN-γ-, IL-17A-, and IL-10-producing T cell production as well as FOXP3$^+$ Treg cells. **(A)** Percentage of CD3$^+$CD4$^+$ T cells producing IFN-γ, IL-10 and IL-17A generated after 3 days of co-culture with control excipients-, V132-, MV140-, and MV140/V132-stimulated hmoDCs as determined by intracellular staining and flow cytometry analysis. Representative dot plots are shown for the intracellular staining after flow cytometry analysis. **(B)** Percentage of induced CD4$^+$CD25^{high}CD127$^-$FOXP3$^+$ Treg cells gated on CD4$^+$ T cells after 3 days of co-culture with allogeneic control excipients-, V132-, MV140-, and MV140/V132-activated hmoDCs. Results are mean ± s.e.m. of 7-8 **(A)**, and 5 **(B)** independent experiments. Paired Student *t* test, $^*P < 0.05$, and $^{**}P < 0.01$.

MV140/V132 Stimulation in Human Dendritic Cells Activates Mitogen-Activated Protein Kinases, Nuclear Factor-κB, and mTOR Signaling Pathways

MV140 triggers CLRs and TLRs in human DCs, which culminate in the activation of mitogen-activated protein kinases (MAPKs)- and nuclear factor-κB (NF-κB)-mediated signaling pathways (26). *C. albicans* activates a complex network of PRRs that also lead to the subsequent activation of MAPKs and NF-κB (44). Our data revealed that V132 rapidly induced activation of the MAPK extracellular signal-regulated kinases 1/2 (ERK1/2) (phosphorylation of Thr202/Tyr204) and the MAPK c-Jun NH_2-terminal kinase (JNK) (phosphorylation of Thr183/Tyr185) in hmoDCs without activation of MAPK p38 (phosphorylation of Thr108/Tyr182) (**Figure 4A**). MV140 alone induced a significantly higher phosphorylation of p38 and IKKα/β than V132 alone in hmoDCs without significant changes observed for ERK or JNK compared to V132 alone (**Figure 4A**). V132 alone induced significant phosphorylation of ERK and JNK compared to unstimulated cells without changes in p38 or IKKα/β. Interestingly, MV140/V132 induced a significantly higher phosphorylation of ERK, p38, and IKKα/β than V132 (**Figure 4A**). However, a slight but non-significant increment in the activation of ERK and JNK was observed after MV140/V132 stimulation compared with MV140 alone without differences in the activation of p38 or IKKα/β (**Figure 4A**). Sensing of *C. albicans* β-glucan by dectin-1 induces the activation of mTOR signaling pathway (31), however, the implication of mTOR pathway in the mechanism of action of MV140 remains unknown. V132, MV140 and MV140/V132 induced the activation of the mTOR pathway as determined by the phosphorylation of the upstream activator Akt (Ser473) and the downstream substrate p70S6 kinase (Thr389) (**Figure 4B**). V132 and MV140/V132 stimulation induces a similar and significantly higher phosphorylation of Akt and p70S6K than MV140 in hmoDCs (**Figure 4B**).

MV140/V132 Induces Metabolic and Epigenetic Reprogramming in Human Dendritic Cells

We studied metabolic changes in hmoDCs stimulated with V132, MV140 or the combination of MV140 and V132. A slight non-significant increase in the consumption of glucose and metabolic rate was observed in hmoDCs after V132 stimulation compared to excipients-treated hmoDCs (**Figure 5A**). In contrast, the stimulation of hmoDCs with MV140 or MV140/V132 significantly enhanced the consumption of glucose from the culture medium and, therefore, the metabolic rate compared to excipients- or V132-treated hmoDCs, without significant differences among them (**Figure 5A**). However, MV140/V132-activated hmoDCs significantly increased lactate production relative to MV140-stimulated hmoDCs, suggesting that the combination of MV140 and V132 is able to enhance Warburg effect in human DCs (**Figure 5B**). In the same line, hmoDCs stimulated with MV140/V132 displayed a significant decrease in the mitochondrial membrane potential relative to

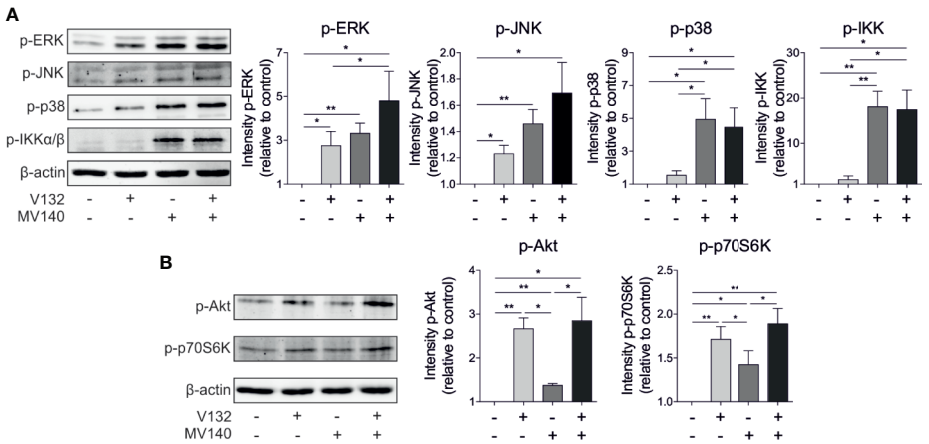

FIGURE 4 | Activation of MAPKs, NF-κB, and mTOR signaling pathways in hmoDCs stimulated with control excipients, V132, MV140, or MV140/V132. **(A, B)** Western blot analysis of protein extracts from hmoDCs stimulated for 30 min in the indicated conditions. Quantification of the reactive phosphorylated bands by scanning densitometry. β-actin was used as a loading control. One representative example is shown. Results are mean ± s.e.m. of four to seven independent experiments. Paired Student *t* test, $^{*}P < 0.05$, and $^{**}P < 0.01$.

stimulation with MV140 alone (**Figure 5C**), supporting that the combination of MV140 and V132 induces a metabolic rewiring that enhances glycolysis and lactic fermentation and reduces mitochondrial activity. Supporting these data, MV140/V132-stimulated hmoDCs showed a significantly lower mitochondrial content than hmoDCs stimulated with MV140 alone (**Figure 5D**) as determined by calculating the ratio of mitochondrial DNA (COXII)/nuclear DNA (GAPDH) as described in (45). Next, we studied the potential implication of the transcription factor HIF-1α as a key regulator of glycolysis and other metabolic processes. No changes in the expression of HIF-1α gene were observed after V132 stimulation compared with control cells. However, both MV140- and MV140/V132-activated hmoDCs expressed significantly higher mRNA levels of HIF-1α than excipients- and V132-treated hmoDCs, without significant differences among them (**Figure 5E**). Metabolic reprogramming has been associated with changes in the epigenome *via* histone methylation and acetylation. To assess whether V132, MV140, and MV140/V132 could induce epigenetic reprogramming in hmoDCs, we performed inhibition experiments using the histone methyltransferase inhibitor 5′-methylthioadenosine (MTA) and the histone demethylase inhibitor pargyline. Interestingly, TNF-α, IL-23, and IL-10 production was significantly impaired in the presence of MTA, but preserved in the presence of pargyline after stimulation of hmoDCs with V132, MV140, or MV140/V132 (**Figure 5F**), suggesting that epigenetic modifications are involved in the increased production of the assayed cytokines. An enhanced IL-10 production was observed in the presence of pargyline compared with stimulated cells without the demethylase inhibitor (**Figure 5F**). Cell viability was not significantly affected in any case and only a slight decrease in cell viability was observed for pargyline after V132 or MV140/V132 stimulation (**Supplementary Figure 2**).

In Vivo Sublingual Immunization of BALB/c Mice With MV140/V132 Enhances T Cell Proliferation and Induces Specific Antibodies Against All the Components in the Formulation

To study the capacity of the different assayed preparations to induce humoral responses *in vivo*, BALB/c mice were sublingually immunized with MV140/V132 or control excipients and the induced T and B cell responses analyzed (**Figure 6A**). Splenic $CD4^+$ T cells from mice immunized with MV140/V132 or control excipients showed significantly higher proliferation rates after *in vitro* stimulation with all the assayed stimuli than after *in vitro* stimulation with control excipients (**Figure 6B**). Remarkably, splenic $CD4^+$ T cells from mice sublingually immunized with MV140/V132 displayed significantly higher proliferation rates than those from control mice after *in vitro* stimulation with V132, MV140, MV140/V132 or with the non-specific T cell mitogen PHA (**Figure 6B**). Mice immunized with MV140/V132 produced significantly higher levels of specific IgG and IgA against all the components of the vaccine formulation including *C. albicans* (V132) and the individual bacterial components of MV140 (*K. pneumoniae*, *E. coli*, *P. vulgaris* and *E. faecalis*) than control mice (**Figures 6C, D**). As shown in **Figure 6D**, the highest specific IgG responses were against the MV140 gram-negative components *E. coli* and *P. vulgaris* whereas for IgA responses the highest levels were observed for *E. faecalis* and *P. vulgaris*. Interestingly, MV140/V132 immunization induced not only specific IgG1 but also IgG2a against all the components of the vaccine formulation (**Supplementary Figure 3**). As shown in this figure, similar specific IgG1 and IgG2a responses were induced upon MV140/V132 immunization, suggesting also the priming of IFN-γ-producing CD4 T cells driving IgG2a isotype switch. Sublingual immunization with MV140/V132 induced higher IgG responses against *E. coli* and *P. vulgaris* than immunization with

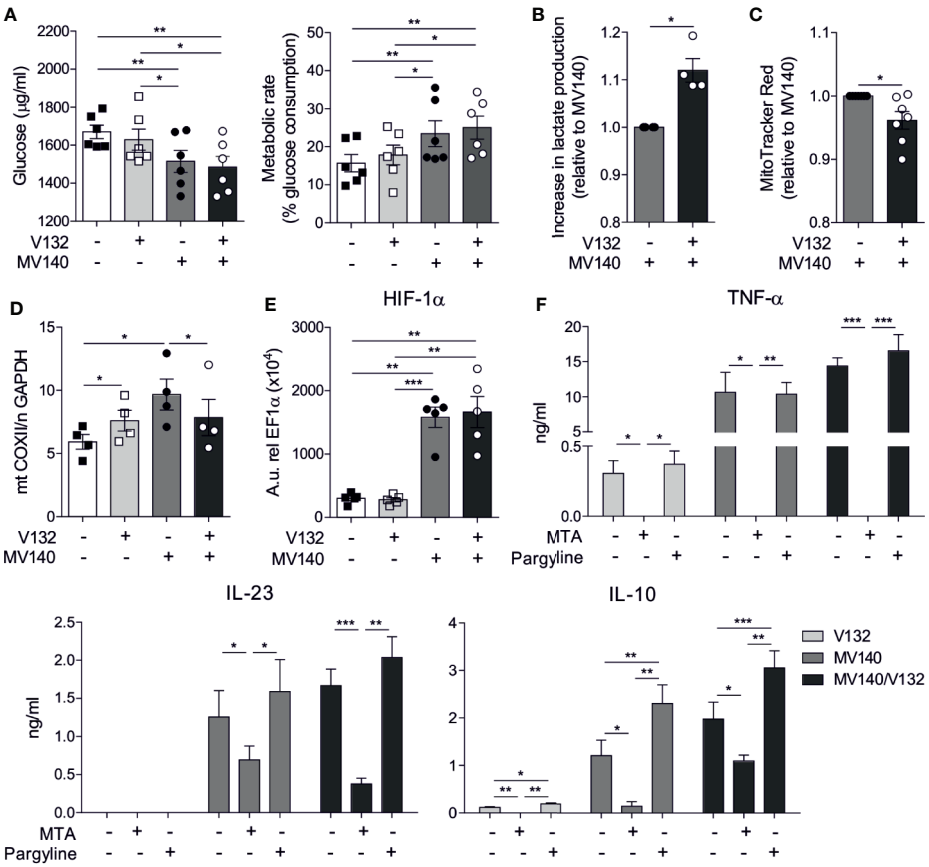

FIGURE 5 | MV140/V132 induces metabolic and epigenetic reprogramming in hmoDCs. **(A)** Glucose consumption by hmoDCs treated with control excipients, V132, MV140 and MV140/V142 after 18 h and calculated metabolic rate. **(B)** Increase in lactate production in cell-free supernatants from MV140/V132-stimulated hmoDCs relative to MV140-activated hmoDCs. **(C)** Relative changes in fluorescence intensity of stimulated hmoDCs stained with MitoTracker Red. **(D)** Mitochondrial mass determination as the ratio of mitochondrial (mt) and nuclear (n) DNA as expressed by mt COXII/n GAPDH. **(E)** Messenger RNA expression levels of HIF-1α gene in hmoDCs stimulated for 18 h. Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4, with ΔCT defined as the difference between the cycle threshold value for the gene of interest and EF1α. **(F)** Cytokine production by V132-, MV140-, and MV140/V132-activated hmoDCs in the presence of 5′-methylthioadenosine (MTA) or pargyline as inhibitors of histone methyltransferases and demethylases, respectively. Results are mean ± s.e.m. of 6 **(A)**, 4 **(B)**, 7 **(C)**, 4 **(D)**, 5 **(E)**, and 4-7 **(F)** independent experiments. Paired Student *t* test, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$.

MV140 alone whereas only a slight increase for *K. pneumoniae* and a slight decrease for *E. faecalis* were observed (**Figure 6E**). MV140/V132 induced higher IgA responses for *K. pneumoniae* and *P. vulgaris* than immunization with MV140 alone whereas lower IgA levels were detected for *E. coli* without changes for *E. faecalis* (**Figure 6E**). Interestingly, *in vivo* sublingually immunization with MV140/V132 induced higher specific IgG and IgA responses against *C. albicans* than immunization with V132 alone, which was special marked for IgA, thus indicating that the incorporation of MV140 into V132 preparations might well significantly increase the production of mucosal IgA antibodies specific for *C. albicans* compared to V132 alone (**Figure 6F**).

DISCUSSION

In this study, we demonstrated that the combination of MV140 and V132 at an equal proportion imprints human DCs with the capacity to polarize potent IFN-γ– and IL-17A–producing T cells and $FOXP3^+$ regulatory T (Treg) cells. MV140/V132 induces a more potent maturation of DCs and a higher capacity to produce pro-inflammatory cytokines and IL-10 than MV140 or V132 alone. Mechanistically, we showed that MV140/V132 activates MAPK-, NF-κB- and mTOR-mediated signaling pathways in human DCs compared to unstimulated cells, which might well be connected to the induced metabolic and epigenetic reprogramming as key molecular mechanisms driving trained immunity. In particular, MV140/V132-activated hmoDCs enhance glycolytic capacity and reduce mitochondrial activity. MV140/V132 stimulation also leads to histone methylation that enhances the production of TNF-α, IL-23, and IL-10 cytokines by human DCs. *In vivo* sublingual immunization of BALB/c mice with MV140/V132 resulted in enhanced proliferative responses of splenic $CD4^+$ T cells not only upon *in vitro* stimulation with the related antigens contained in the vaccine formulation but also upon stimulation with phytohaemagglutinin, a non-specific

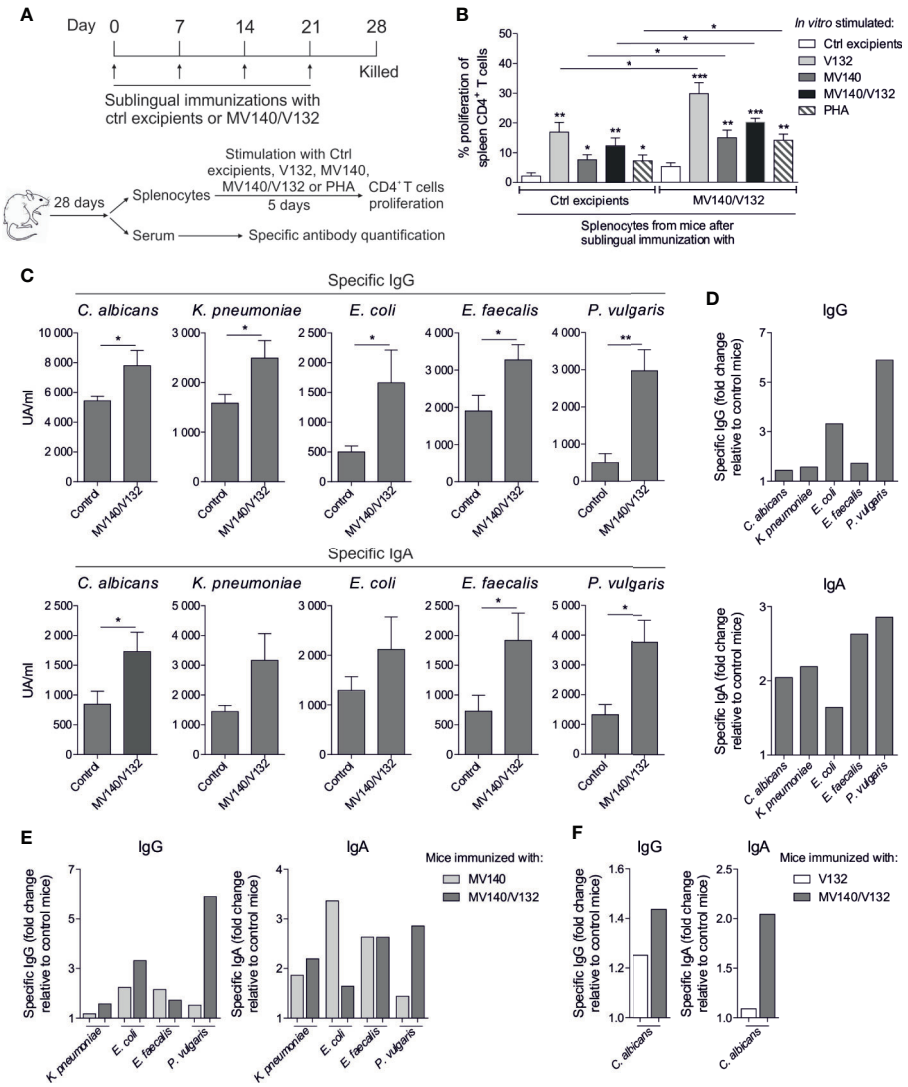

FIGURE 6 | Sublingual immunization of BALB/c mice with MV140/V132 enhances splenic T cell responses and induces specific antibodies against all the components included in the formulation. **(A)** Scheme of the sublingual immunization protocol and analysis of induced systemic responses. **(B)** Proliferation of CFSE-labeled $CD4^+$ T cells from splenocytes isolated from mice immunized sublingually with MV140/V132 or control excipients after *in vitro* stimulation with V132, MV140, V140/V132, phytohaemagglutinin (PHA), or control (containing all excipients except the bacteria and yeast). **(C)** Serum IgG and IgA antibodies specific for **(C)** *albicans*, *K pneumoniae*, *E coli*, *E faecalis* and *P. vulgaris* from mice immunized with MV140/V132 or with control excipients. **(D)** Fold change of specific IgG and IgA antibodies generated in mice immunized with MV140/V132 relative to control mice. **(E)** Fold change of specific IgG and IgA antibodies for *K pneumoniae*, *E coli*, *E faecalis* and *P. vulgaris* generated in mice immunized with MV140/V132 or MV140 alone relative to control mice. **(F)** Fold change of specific IgG and IgA antibodies for *C albicans* generated in mice immunized with MV140/V132 or V132 alone relative to control mice. Results are mean ± s.e.m. of 7-8 **(B)** and 5-6 **(C–F)** individual mice per condition of one single experiment. Unpaired *t* test, $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

T cell mitogen, which suggests the induction of trained immunity mechanisms. Remarkably, *in vivo* sublingual immunization with MV140/V132 induces the generation of specific IgG and IgA antibodies against all the components contained in the vaccine formulation. Overall, we report for the first time immunological mechanisms underlying the potential mode of action of a combination of MV140 and V132 as a novel promising trained immunity-based vaccine for GUTIs.

MV140 (Uromune) is a sublingual vaccine for RUTIs prophylaxis that is able to prime human DCs with the capacity to induce Th1-, Th17-, and IL-10–producing T cells (26). Clinical studies have demonstrated that MV140 significantly prevents RUTIs in 80% to 90% of individuals during the year following vaccination (39–43). The reported clinical benefits indicate that sublingual administration of MV140 also reduces the overuse of antibiotics, which not only might reduce the increase in antibiotic-resistances (40), but also avoid microbiota disruption (1, 7, 9–12). Due to the high incidence of RVVCs and the difficulties for their treatment, mainly based on antifungals, new approaches to combat these infections are also fully

demanded. Among these alternatives, classical anti-*C. albicans* vaccines based on recombinant proteins or cell wall-derived glycans to induce specific protective antibodies have been developed (46–48). However, up to date no safe and effective antifungal vaccine against RVVCs is still available (25). At this regard, V132 is a mucosal heat-inactivated *C. albicans* vaccine that could represent a suitable strategy to improve treatments for RVVCs. A small pilot study performed by our groups showed that sublingual administration of V132 significantly prevented VVCs recurrence, conferring additional protection to RUTIs when combined at 50% with MV140 in the formulation (unpublished data). Supporting these observations, it was previously reported that intranasal immunization with heat-inactivated *C. albicans* generates protection against *C. albicans* infection in mice and, interestingly, the protective role of this fungal vaccine was higher when mice were immunized with *C. albicans* in combination with *E. coli* toxin as a mucosal adjuvant than the single heat-inactivated fungal administration (49). As RUTIs and RVVCs are related to each other, a polymicrobial formulation combining both MV140 and V132 might well be an alternative approach for the treatment of GUTIs that could improve the efficacy of the vaccination within a single preparation. However, prior to definitive translation into clinical practice further preclinical mechanistic studies are needed. The addition of V132 to MV140 would incorporate *C. albicans* antigens into the vaccine, which could induce antigen-specific responses also against the yeast. In addition, our *in vivo* data indicated that mice immunized with MV140/V132 displayed stronger IgG and IgA responses against *C. albicans* than those immunized with V132 alone, suggesting that MV140 might well enhance the capacity of V132 to induce humoral responses. However, how the combination of V132 and MV140 might regulate mucosal DCs' function and their capacity to prime adaptive immune responses at the molecular level remained fully elusive.

Our data revealed that MV140/V132 promotes a potent pro-inflammatory cytokine production (TNF-α and IL-23) and higher levels of IL-10 in human DCs compared with individual MV140 or V132 stimulation (26), which are key cytokines also to polarize T cell responses (50, 51). Human DCs activated with MV140/V132 generate potent IFN-γ– and IL-17A–producing T cells that could contribute to eradicate intracellular and extracellular pathogens, respectively (51). MV140/V132 also induces higher frequencies of IL-10–producing T cells and $FOXP3^+$ Treg cells than individual MV140 and V132, which are essential to control excessive immune responses, to enhance microbiota protection and tissue repair after long-term antibiotic treatments and play a key role in restoration of healthy immune responses in urogenital infections (50, 52–54). Additionally, *in vivo* sublingual immunization with MV140/V132 induced the generation of significant levels of specific IgG and IgA antibodies against all the components contained in the vaccine formulation, which might well confer protection against infections caused by these specific pathogens (33, 55). It is also noteworthy to highlight that splenic $CD4^+$ T cells from mice that were sublingually immunized with MV140/V132 displayed significantly higher proliferation rates than those from control mice not only after *in vitro* stimulation with the vaccine components (either separately or in combination) but also after *in vitro* stimulation with the non-specific T cell mitogen PHA. These data suggest that MV140/V132 immunization might well promote trained immunity mechanisms that enhance both innate and adaptive immune responses against a broad range of pathogens, contained or not in the vaccine formulation, upon new encounters. At this regard, metabolic and epigenetic reprogramming represent well-recognized mechanisms underlying trained immunity in myeloid cells, including DCs (23). The regulation of metabolic rewiring plays an important role in the immunogenic vs tolerogenic properties of DCs (35, 36). For example, the switch to glycolysis and lactic fermentation couple to anabolic metabolism for the biosynthesis needed for cell growth is associated with DCs activation (35, 56). We showed that MV140/V132-activated hmoDCs display enhanced metabolic rate and lactate production while reduced mitochondrial activity, suggesting Warburg effect induction. Supporting this observation MV140/V132 not only induced the rapid activation of immune pathways involved in cytokine and surface marker production such as those mediated by MAPKs or NK-κB but also mTOR-mediated signaling pathways as a central regulator of metabolic reprogramming in hmoDCs (35, 57, 58). mTOR controls numerous cellular processes in DCs to adapt the cellular metabolism to immune functions such as antigen presentation or cytokine production (35, 59). Interestingly, V132 alone and MV140/V132 stimulation induced a higher activation of mTOR signaling pathway than MV140 alone, indicating that V132 significantly contributes to the activation of this key signaling pathway in the induction of innate trained immunity mechanisms. In addition, the activation of the MAPK JNK and p38 are also relevant for the induction of trained immunity in human monocytes (60). Our data show that MV140 is the main driver of p38 and JNK within the MV140/V132 formulation, suggesting also an important contribution of these MV140-induced pathways in the induction of trained immunity. HIF-1α activation is involved in enhanced glycolytic activity and lactate production by the induction of metabolism-related genes (61). MV140 alone and MV140/V132 but not V132 alone enhance HIF-1α mRNA expression levels in hmoDCs. These data suggest that the metabolic reprogramming induced by MV140/V132 might be due to the contribution of both HIF-1α expression, enhanced by bacterial components (MV140) of the preparation (61), and mTOR signaling, induced by *C. albicans* (V132) (31), which together might well drive a more potent glycolytic metabolism. Changes in cellular metabolism could be also accompanied by epigenetic reprogramming, mainly *via* methylation (32, 62). Activation of immune cells induces histone modifications affecting the gene expression patterns (32, 63). The methyltransferase inhibitor MTA but not the histone demethylase inhibitor pargyline abolishes V132-, MV140- and MV140/V132-induced cytokine production in hmoDCs, suggesting that histone methylation is an epigenetic change essential in the mechanism of action of these stimuli (30, 60).

Collectively, MV140/V132-stimulated human DCs activate the Akt/mTOR/HIF-1α axis leading to metabolic and epigenetic

reprogramming, which are defining hallmarks of trained immunity. These mechanistic insights might well support the trained immunity effects observed after sublingually immunization of BALB/c mice with MV140/V132 (23, 29–32, 62, 64). In conclusion, the combination of MV140 and V132 in a single vaccine formulation could represent a novel promising TIbV for RUTIs and RVVCs. In the long run and upon proper clinical translations, this study might well pave the way for the development of novel alternative approaches for GUTIs.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are readily available upon request. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Host Institution UCM. Fully anonymous Buffy Coats from healthy donors were provided by the "Centro de Transfusión de la Comunidad de Madrid". UCM is perfectly equipped with the adequate biosecurity infrastructure required to carry out the research presented in this proposal. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Biolab S.L. Animals were maintained in Biolab S.L. under the conditions stabilized by the R.D. 53/2013 and in accordance with the international GLP normative, RD 223/83 and ISO 10993-2:2007. The tests are performed in accordance with official methods, European Pharmacopoeia, USP Pharmacopoeia, OECD Guideline, UNE EN-ISO, and specific methodologies developed for R&D.

AUTHOR CONTRIBUTIONS

OP conceived and designed the study. LM-C, CS-O (human experiments), C B-V (technical support for human experiments), and CD-R (mice experiments) performed the experiments. SS-R, JLS, and OP provided the reagents. LM-C, CS-O, CB-V, C.M. D-R, SS-R, JLS, and OP analyzed and discussed the data. OP and L.M-C wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grant SAF-2017-84978-R to OP from MINECO, Spain, and by unrestricted grant from Inmunotek under an Art.83 UCM contract to OP. LM-C and CB-V are recipients of FPU predoctoral fellowships from MINECO, Spain. CS-O is recipient of a predoctoral fellowship under an Industrial Doctorate Program (UCM-Inmunotek S.L) sponsored by CAM, Spain.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.612269/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Production of TGF-β1 and expression of CD86 and HLA-DR in hmoDCs after control excipients, V132, MV140, and MV140/V132 stimulation. **(A)** TGF-β1 production after stimulation of hmoDCs with control excipients, V132, MV140 or MV140/V132 for 18 h quantified in cell-free supernatants by ELISA**. (B)** Flow cytometry representative dot plots for the expression of surface markers HLA-DR and CD86 after stimulation of hmoDCs with control, V132, MV140 and MV140/V132 for 18 h. **(C)** Quantification of the percentage of positive cells and **(D)** Mean Fluorescence Intensity (MFI) values for CD86 after stimulation of hmoDCs. Results are mean ± s.e.m. of 8 **(B, C)** independent experiments. Paired Student *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

SUPPLEMENTARY FIGURE 2 | Percentage of viability for each assayed condition respect to each vehicle control by using trypan blue exclusion. Results are mean ± s.e.m. of 3-4 independent experiments.

SUPPLEMENTARY FIGURE 3 | (A) Serum IgG1 and IgG2a antibodies specific for *C. albicans*, *K. pneumoniae*, *E. coli*, *E. faecalis* and *P. vulgaris* from mice immunized with MV140/V132 or with control excipients. **(B)** Fold change of specific IgG1 and IgG2a antibodies generated in mice immunized with MV140/V132 relative to control mice. Results are mean ± s.e.m. of 4-5 individual mice per condition of one independent experiments. Unpaired t test, $^{*}P < 0.05$.

REFERENCES

1. Klein RD, Hultgren SJ. Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. *Nat Rev Microbiol* (2020) 18(4):211–26. doi: 10.1038/s41579-020-0324-0
2. Medina M, Castillo-Pino E. An introduction to the epidemiology and burden of urinary tract infections. *Ther Adv Urol* (2019) 11:1756287219832172. doi: 10.1177/1756287219832172
3. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nat Rev Microbiol* (2015) 13(5):269–84. doi: 10.1038/nrmicro3432
4. Foxman B. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect Dis Clin North Am* (2014) 28(1):1–13. doi: 10.1016/j.idc.2013.09.003
5. Dason S, Dason JT, Kapoor A. Guidelines for the diagnosis and management of recurrent urinary tract infection in women. *Can Urol Assoc J* (2011) 5 (5):316–22. doi: 10.5489/cuaj.11214
6. Laxminarayan R, Van Boeckel T, Frost I, Kariuki S, Khan EA, Limmathurotsakul D, et al. The Lancet Infectious Diseases Commission on antimicrobial resistance: 6 years later. *Lancet Infect Dis* (2020) 20(4):e51–60. doi: 10.1016/S1473-3099(20)30003-7
7. Macklaim JM, Clemente JC, Knight R, Gloor GB, Reid G. Changes in vaginal microbiota following antimicrobial and probiotic therapy. *Microb Ecol Health Dis* (2015) 26:27799. doi: 10.3402/mehd.v26.27799
8. Goncalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S. Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit Rev Microbiol* (2016) 42(6):905–27. doi: 10.3109/1040841X.2015.1091805

9. Shukla A, Sobel JD. Vulvovaginitis Caused by Candida Species Following Antibiotic Exposure. *Curr Infect Dis Rep* (2019) 21(11):44. doi: 10.1007/s11908-019-0700-y
10. Xu J, Schwartz K, Bartoces M, Monsur J, Severson RK, Sobel JD. Effect of antibiotics on vulvovaginal candidiasis: a MetroNet study. *J Am Board Fam Med* (2008) 21(4):261–8. doi: 10.3122/jabfm.2008.04.070169
11. McCool L, Mai H, Essmann M, Larsen B. Tetracycline effects on Candida albicans virulence factors. *Infect Dis Obstet Gynecol* (2008) 2008:493508. doi: 10.1155/2008/493508
12. Wilton L, Kollarova M, Heeley E, Shakir S. Relative risk of vaginal candidiasis after use of antibiotics compared with antidepressants in women: postmarketing surveillance data in England. *Drug Saf* (2003) 26(8):589–97. doi: 10.2165/00002018-200326080-00005
13. Sobel JD. Vulvovaginal candidosis. *Lancet* (2007) 369(9577):1961–71. doi: 10.1016/S0140-6736(07)60917-9
14. Sobel JD. Recurrent vulvovaginal candidiasis. *Am J Obstet Gynecol* (2016) 214(1):15–21. doi: 10.1016/j.ajog.2015.06.067
15. Guzel AB, Ilkit M, Akar T, Burgut R, Demir SC. Evaluation of risk factors in patients with vulvovaginal candidiasis and the value of chromID Candida agar versus CHROMagar Candida for recovery and presumptive identification of vaginal yeast species. *Med Mycol* (2011) 49(1):16–25. doi: 10.3109/13693786.2010.497972
16. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R. Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* (2018) 18(11):e339–e47. doi: 10.1016/S1473-3099(18)30103-8
17. Yano J, Sobel JD, Nyirjesy P, Sobel R, Williams VL, Yu Q, et al. Current patient perspectives of vulvovaginal candidiasis: incidence, symptoms, management and post-treatment outcomes. *BMC Womens Health* (2019) 19(1):48. doi: 10.1186/s12905-019-0748-8
18. Crouss T, Sobel JD, Smith K, Nyirjesy P. Long-Term Outcomes of Women With Recurrent Vulvovaginal Candidiasis After a Course of Maintenance Antifungal Therapy. *J Low Genit Tract Dis* (2018) 22(4):382–6. doi: 10.1097/LGT.0000000000000413
19. Marchaim D, Lemanek L, Bheemreddy S, Kaye KS, Sobel JD. Fluconazole-resistant Candida albicans vulvovaginitis. *Obstet Gynecol* (2012) 120(6):1407–14. doi: 10.1097/AOG.0b013e31827307b2
20. Wagenlehner F, Wullt B, Ballarini S, Zingg D, Naber KG. Social and economic burden of recurrent urinary tract infections and quality of life: a patient web-based study (GESPRIT). *Expert Rev Pharmacoecon Outcomes Res* (2018) 18(1):107–17. doi: 10.1080/14737167.2017.1359543
21. Fukazawa EI, Witkin SS, Robial R, Vinagre JG, Baracat EC, Linhares IM. Influence of recurrent vulvovaginal candidiasis on quality of life issues. *Arch Gynecol Obstet* (2019) 300(3):647–50. doi: 10.1007/s00404-019-05228-3
22. Loubet P, Ranfaing J, Dinh A, Dunyach-Remy C, Bernard L, Bruyere F, et al. Alternative Therapeutic Options to Antibiotics for the Treatment of Urinary Tract Infections. *Front Microbiol* (2020) 11:1509. doi: 10.3389/fmicb.2020.01509
23. Sanchez-Ramon S, Conejero L, Netea MG, Sancho D, Palomares O, Subiza JL. Trained Immunity-Based Vaccines: A New Paradigm for the Development of Broad-Spectrum Anti-infectious Formulations. *Front Immunol* (2018) 9:2936. doi: 10.3389/fimmu.2018.02936
24. Tejera-Alhambra M, Palomares O, Perez de Diego R, Diaz-Lezcano I, Sanchez-Ramon S. New Biological Insights in the Immunomodulatory Effects of Mucosal Polybacterial Vaccines in Clinical Practice. *Curr Pharm Des* (2016) 22(41):6283–93. doi: 10.2174/1381612822666160829143129
25. Tso GHW, Reales-Calderon JA, Pavelka N. The Elusive Anti-Candida Vaccine: Lessons From the Past and Opportunities for the Future. *Front Immunol* (2018) 9:897. doi: 10.3389/fimmu.2018.00897
26. Benito-Villalvilla C, Cirauqui C, Diez-Rivero CM, Casanovas M, Subiza JL, Palomares O. MV140, a sublingual polyvalent bacterial preparation to treat recurrent urinary tract infections, licenses human dendritic cells for generating Th1, Th17, and IL-10 responses via Syk and MyD88. *Mucosal Immunol* (2017) 10(4):924–35. doi: 10.1038/mi.2016.112
27. Cirauqui C, Benito-Villalvilla C, Sanchez-Ramon S, Sirvent S, Diez-Rivero CM, Conejero L, et al. Human dendritic cells activated with MV130 induce Th1, Th17 and IL-10 responses via RIPK2 and MyD88 signalling pathways. *Eur J Immunol* (2018) 48(1):180–93. doi: 10.1002/eji.201747024
28. Guevara-Hoyer K, Saz-Leal P, Diez-Rivero CM, Ochoa-Grullon J, Fernandez-Arquero M, Perez de Diego R, et al. Trained Immunity Based-Vaccines as a Prophylactic Strategy in Common Variable Immunodeficiency. A Proof of Concept Study. *Biomedicines* (2020) 8(7):203. doi: 10.3390/biomedicines8070203
29. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe* (2011) 9(5):355–61. doi: 10.1016/j.chom.2011.04.006
30. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* (2012) 12(2):223–32. doi: 10.1016/j.chom.2012.06.006
31. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* (2014) 345(6204):1250684. doi: 10.1126/science.1250684
32. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science* (2014) 345(6204):1251086. doi: 10.1126/science.1251086
33. Lamichhane A, Azegamia T, Kiyonoa H. The mucosal immune system for vaccine development. *Vaccine* (2014) 32(49):6711–23. doi: 10.1016/j.vaccine.2014.08.089
34. Palomares O, Akdis M, Martin-Fontecha M, Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunol Rev* (2017) 278(1):219–36. doi: 10.1111/imr.12555
35. Wculek SK, Khouili SC, Priego E, Heras-Murillo I, Sancho D. Metabolic Control of Dendritic Cell Functions: Digesting Information. *Front Immunol* (2019) 10:775. doi: 10.3389/fimmu.2019.00775
36. O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol* (2016) 16(9):553–65. doi: 10.1038/nri.2016.70
37. Benito-Villalvilla C, Soria I, Perez-Diego M, Fernandez-Caldas E, Subiza JL, Palomares O. Alum impairs tolerogenic properties induced by allergoid-mannan conjugates inhibiting mTOR and metabolic reprogramming in human DCs. *Allergy* (2020) 75(3):648–59. doi: 10.1111/all.14036
38. Nickel JC, Saz-Leal P, Doiron RC. Could sublingual vaccination be a viable option for the prevention of recurrent urinary tract infection in Canada? A systematic review of the current literature and plans for the future. *Can Urol Assoc J* (2020) 14(8):281–7. doi: 10.5489/cuaj.6690
39. Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Criado FJ, Miron-Canelo JA, Gil-Vicente A, Nieto-Huertos A, et al. Evaluation of a therapeutic vaccine for the prevention of recurrent urinary tract infections versus prophylactic treatment with antibiotics. *Int Urogynecol J* (2013) 24(1):127–34. doi: 10.1007/s00192-012-1853-5
40. Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Cenador MB, Virseda-Rodriguez AJ, Martin-Garcia I, Sanchez-Escudero A, et al. Comparison of sublingual therapeutic vaccine with antibiotics for the prophylaxis of recurrent urinary tract infections. *Front Cell Infect Microbiol* (2015) 5:50. doi: 10.3389/fcimb.2015.00050
41. Yang B, Foley S. First experience in the UK of treating women with recurrent urinary tract infections with the bacterial vaccine Uromune((R)). *BJU Int* (2018) 121(2):289–92. doi: 10.1111/bju.14067
42. Ramirez Sevilla C, Gomez Lanza E, Manzanera JL, Martin JAR, Sanz MAB. Active immunoprophyilaxis with uromune(R) decreases the recurrence of urinary tract infections at three and six months after treatment without relevant secondary effects. *BMC Infect Dis* (2019) 19(1):901. doi: 10.1186/s12879-019-4541-y
43. Carrion-Lopez P, Martinez-Ruiz J, Libran-Garcia L, Gimenez-Bachs JM, Pastor-Navarro H, Salinas-Sanchez AS. Analysis of the Efficacy of a Sublingual Bacterial Vaccine in the Prophylaxis of Recurrent Urinary Tract Infection. *Urol Int* (2020) 104(3-4):293–300. doi: 10.1159/000505162
44. Netea MG, Brown GD, Kullberg BJ, Gow NA. An integrated model of the recognition of Candida albicans by the innate immune system. *Nat Rev Microbiol* (2008) 6(1):67–78. doi: 10.1038/nrmicro1815
45. Malinarich F, Duan K, Hamid RA, Bijin A, Lin WX, Poidinger M, et al. High mitochondrial respiration and glycolytic capacity represent a metabolic

phenotype of human tolerogenic dendritic cells. *J Immunol* (2015) 194 (11):5174–86. doi: 10.4049/jimmunol.1303316
46. Edwards JEJr., Schwartz MM, Schmidt CS, Sobel JD, Nyirjesy P, Schodel F, et al. A Fungal Immunotherapeutic Vaccine (NDV-3A) for Treatment of Recurrent Vulvovaginal Candidiasis-A Phase 2 Randomized, Double-Blind, Placebo-Controlled Trial. *Clin Infect Dis* (2018) 66(12):1928–36. doi: 10.1093/cid/ciy185
47. De Bernardis F, Amacker M, Arancia S, Sandini S, Gremion C, Zurbriggen R, et al. A virosomal vaccine against candidal vaginitis: immunogenicity, efficacy and safety profile in animal models. *Vaccine* (2012) 30(30):4490–8. doi: 10.1016/j.vaccine.2012.04.069
48. Torosantucci A, Bromuro C, Chiani P, De Bernardis F, Berti F, Galli C, et al. A novel glyco-conjugate vaccine against fungal pathogens. *J Exp Med* (2005) 202 (5):597–606. doi: 10.1084/jem.20050749
49. Cardenas-Freytag L, Cheng E, Mayeux P, Domer JE, Clements JD. Effectiveness of a vaccine composed of heat-killed Candida albicans and a novel mucosal adjuvant, LT(R192G), against systemic candidiasis. *Infect Immun* (1999) 67(2):826–33. doi: 10.1128/IAI.67.2.826-833.1999
50. Akdis M, Aab A, Altunbulakli C, Azkur K, Costa RA, Crameri R, et al. Interleukins (from IL-1 to IL-38), interferons, transforming growth factor beta, and TNF-alpha: Receptors, functions, and roles in diseases. *J Allergy Clin Immunol* (2016) 138(4):984–1010. doi: 10.1016/j.jaci.2016.06.033
51. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol* (2016) 16(3):149–63. doi: 10.1038/nri.2015.18
52. Palomares O, Martin-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, et al. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. *Genes Immun* (2014) 15(8):511–20. doi: 10.1038/gene.2014.45
53. Soerens AG, Da Costa A, Lund JM. Regulatory T cells are essential to promote proper CD4 T-cell priming upon mucosal infection. *Mucosal Immunol* (2016) 9(6):1395–406. doi: 10.1038/mi.2016.19
54. Pandiyan P, Bhaskaran N, Zou M, Schneider E, Jayaraman S, Huehn J. Microbiome Dependent Regulation of Tregs and Th17 Cells in Mucosa. *Front Immunol* (2019) 10:426. doi: 10.3389/fimmu.2019.00426
55. Boyaka PN. Inducing Mucosal IgA: A Challenge for Vaccine Adjuvants and Delivery Systems. *J Immunol* (2017) 199(1):9–16. doi: 10.4049/jimmunol.1601775
56. O'Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med* (2016) 213(1):15–23. doi: 10.1084/jem.20151570
57. Ferreira GB, Vanherwegen AS, Eelen G, Gutierrez ACF, Van Lommel L, Marchal K, et al. Vitamin D3 Induces Tolerance in Human Dendritic Cells by Activation of Intracellular Metabolic Pathways. *Cell Rep* (2015) 10(5):711–25. doi: 10.1016/j.celrep.2015.01.013
58. Fliesser M, Morton CO, Bonin M, Ebel F, Hunniger K, Kurzai O, et al. Hypoxia-inducible factor 1alpha modulates metabolic activity and cytokine release in anti-Aspergillus fumigatus immune responses initiated by human dendritic cells. *Int J Med Microbiol* (2015) 305(8):865–73. doi: 10.1016/j.ijmm.2015.08.036
59. Sukhbaatar N, Hengstschlager M, Weichhart T. mTOR-Mediated Regulation of Dendritic Cell Differentiation and Function. *Trends Immunol* (2016) 37 (11):778–89. doi: 10.1016/j.it.2016.08.009
60. Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, et al. Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. *Clin Vaccine Immunol* (2014) 21(4):534–45. doi: 10.1128/CVI.00688-13
61. Jantsch J, Chakravortty D, Turza N, Prechtel AT, Buchholz B, Gerlach RG, et al. Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function. *J Immunol* (2008) 180(7):4697–705. doi: 10.4049/jimmunol.180.7.4697
62. Arts RJ, Joosten LA, Netea MG. Immunometabolic circuits in trained immunity. *Semin Immunol* (2016) 28(5):425–30. doi: 10.1016/j.smim.2016.09.002
63. Tserel L, Kolde R, Rebane A, Kisand K, Org T, Peterson H, et al. Genome-wide promoter analysis of histone modifications in human monocyte-derived antigen presenting cells. *BMC Genomics* (2010) 11:642. doi: 10.1186/1471-2164-11-642
64. Mulder WJM, Ochando J, Joosten LAB, Fayad ZA, Netea MG. Therapeutic targeting of trained immunity. *Nat Rev Drug Discov* (2019) 18(7):553–66. doi: 10.1038/s41573-019-0025-4

Conflict of Interest: OP has received fee for lectures or participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, Diater, GSK, Inmunotek SL, Novartis, Sanofi Genzyme, Stallergenes and Regeneron. OP has received research grants from Inmunotek SL and Novartis SL. JS is the founder and CEO of Inmunotek SL.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SI declared a shared affiliation, with no collaboration, with several of the authors, LC, CO, CV and OP, to the handling editor at the time of review.

Copyright © 2021 Martin-Cruz, Sevilla-Ortega, Benito-Villalvilla, Diez-Rivero, Sanchez-Ramón, Subiza and Palomares. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 24 November 2022
DOI 10.3389/fimmu.2022.1066383

Check for updates

OPEN ACCESS

EDITED BY
Carlos Pelleschi Taborda,
University of São Paulo, Brazil

REVIEWED BY
Antonio Cassone,
Genetics and Biology (Polo GGB), Italy
Sebastian Wurster,
University of Texas MD Anderson Cancer Center, United States
Paula Sampaio,
University of Minho, Portugal

*CORRESPONDENCE
Oscar Palomares
oscar.palomares@quim.ucm.es

†These authors share last authorship

SPECIALTY SECTION
This article was submitted to Vaccines and Molecular Therapeutics, a section of the journal Frontiers in Immunology

RECEIVED 10 October 2022
ACCEPTED 09 November 2022
PUBLISHED 24 November 2022

CITATION
Martín-Cruz L, Angelina A, Baydemir I, Bulut Ö, Subiza JL, Netea MG, Domínguez-Andrés J and Palomares O (2022) *Candida albicans* V132 induces trained immunity and enhances the responses triggered by the polybacterial vaccine MV140 for genitourinary tract infections. *Front. Immunol.* 13:1066383. doi: 10.3389/fimmu.2022.1066383

COPYRIGHT
© 2022 Martín-Cruz, Angelina, Baydemir, Bulut, Subiza, Netea, Domínguez-Andrés and Palomares. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Candida albicans V132 induces trained immunity and enhances the responses triggered by the polybacterial vaccine MV140 for genitourinary tract infections

Leticia Martín-Cruz[1], Alba Angelina[1], Ilayda Baydemir[2], Özlem Bulut[2], José Luis Subiza[3], Mihai G. Netea[2,4], Jorge Domínguez-Andrés[2†] and Oscar Palomares[1*†]

[1]Department of Biochemistry and Molecular Biology, School of Chemistry, Complutense University of Madrid, Madrid, Spain, [2]Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Centre, Nijmegen, Netherlands, [3]Inmunotek, Alcalá de Henares, Madrid, Spain, [4]Department of Immunology and Metabolism, Life and Medical Sciences Institute, University of Bonn, Bonn, Germany

Introduction: Recurrent urinary tract infections (RUTIs) and recurrent vulvovaginal candidiasis (RVVCs) represent major healthcare problems all over the world. Antibiotics and antifungals are widely used for such infectious diseases, which is linked with microbial resistances and microbiota deleterious effects. The development of novel approaches for genitourinary tract infections (GUTIs) such as trained immunity-based vaccines (TIbV) is therefore highly required. MV140 is a sublingual whole-cell heat-inactivated polybacterial preparation with demonstrated clinical efficacy for RUTIs. The sublingual heat-inactivated *Candida albicans* vaccine V132 has been developed for RVVCs. We previously showed that the combination of MV140 and V132 promotes potent Th1/Th17 and regulatory T-cell responses against antigens contained in the formulation and unrelated antigens. The specific contribution of each preparation to such effects and the underlying molecular mechanisms remain incompletely understood.

Methods: PBMC and monocytes were isolated from healthy donors and *in vitro* stimulated with V132, MV140 or MV140/V132. After 6 days of resting, cells were reestimulated with LPS and MV140. Analysis of cytokine production by ELISA, Seahorse assays for functional metabolic experiments and chromatin immunoprecipitation assays were performed. BALB/c mice were intraperitoneally and sublingually immunized with V132.

Results: We uncover that V132 induces trained immunity in human PBMCs and purified monocytes, significantly increasing the responses triggered by

10.3389/fimmu.2022.1066383

subsequent stimulation with MV140. Mechanistically, V132 drives metabolic rewiring towards increased glycolysis and oxidative phosphorylation and induces epigenetic reprogramming that enhances the transcription of the pro-inflammatory genes *IL6* and *TNFA*. Splenocytes and peritoneal cells from V132-immunize mice show increased responses upon *in vitro* stimulation with MV140. Remarkably, splenocytes from sublingually V132-immunized and MV140 *in vivo* treatment mice show stronger Th17 responses than mice exposed to excipients upon *in vitro* stimulation with MV140.

Conclusion: Overall, we provide novel mechanistic insights into how V132-induced trained immunity enhances both innate and adaptive immune responses triggered by MV140, which might open the door for new interventions for GUTIs with important clinical implications.

KEYWORDS

trained immunity, genito urinary infections, candida albicans V132, polybacterial preparation MV140, metabolic and epigenetic reprogramming

Introduction

Urinary tract infections (UTIs) constitute one of the most prevalent bacterial infections, representing a major healthcare problem of increasing prevalence worldwide, especially in women (1). Despite antibiotic treatments, more than 30% of women experience recurrent UTIs (RUTIs), defined as more than three infections within 12 months (2, 3). Long-term treatment with antibiotics might favor the growth of drug-resistant microorganisms as well as microbiota alterations in the gastrointestinal tract and vagina (4, 5). These alterations facilitate the aberrant proliferation of yeasts such as *Candida albicans*, which is associated with vulvovaginal candidiasis (VVCs) (6). In this regard, around 75% of women experience one episode of VVC during their lifetime and up to 5% develop recurrent episodes (RVVCs) (7–10). Topical and oral antifungal formulations are the standard prescribed treatments (11, 12), hence repeated and prolonged antifungal therapy also increases the risk to develop resistances of the pathogens to the available medication (13, 14).

Mucosal bacterial and fungal vaccines have been suggested as promising alternative strategies for genitourinary tract infections (GUTIs) (15–18). Some of these preparations containing live-attenuated or inactivated pathogens are trained immunity-based vaccines (TIbV), which might confer broad protection against target infections while also exhibiting heterologous effects (19–24). Trained immunity is the process by which a primary stimulus reprogram the function of innate immune cells favoring an augmented response against an either related or unrelated secondary challenge (25, 26). Induction of trained immunity was initially described in monocytes, macrophages and NK cells, but other cell types such as neutrophils, dendritic cells (DCs), innate lymphoid cells and bone marrow progenitors can also display a trained immunity phenotype (27).

Metabolic and epigenetic reprograming are the hallmarks of trained immunity. Metabolism play a major role in the induction and preservation of trained immunity (28, 29). In particular, activation of trained cells is connected with different metabolic pathways connecting the energetic need and the functional activity. An increased glycolysis and oxidative phosphorylation with a higher glucose consumption and production of lactate and ATP are the main cellular metabolic pathways involved in trained immunity (30–33). Several metabolites are fundamental to activate and modulate the epigenetic remodeling in trained cells (29). To facilitate an enhanced responsiveness upon a secondary stimulus, trained cells modify chromatin structure and accessibility of promoters of genes codifying for pro-inflammatory cytokines such as TNFα or IL-6, and glycolytic enzymes (29, 32).

Abbreviations: Akt, protein kinase B; APC, allophycocyanin; BCG, *Bacille Calmette-Guérin*; Ctrl, control excipients; DCs, dendritic cells; ECAR, extracellular acidification rate; FITC, fluorescein isothiocyanate; FTU, Formazine Turbidity Units; GUTIs, genitourinary tract infections; H3K27ac, histone 3 acetylation of lysine 27; H3K9me3, histone 3 trimethylation of lysine 9; HIF-1a, hypoxia inducible factor-1a; mTOR, mammalian target of rapamycin; OCR, oxygen consumption rate OCR; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein; RUTIs, recurrent urinary tract infections; RVVCs, recurrent vulvovaginal candidiasis; TIbV, trained immunity-based vaccines; Treg, regulatory T; UTIs, urinary tract infections; VVCs, vulvovaginal candidiasis.

MV140 (Uromune) is a whole-cell, heat-inactivated, sublingual bacterial preparation containing equal proportions of those bacteria causing most of RUTIs across Europe (*Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Enterococcus faecalis*) (21). This sublingual vaccine significantly reduces the infection rates in patients suffering from RUTIs (34–41). In addition, MV140 induces a potent activation of human DCs and the generation of Th1, Th17 and regulatory T (Treg) cells (21, 22). On the other hand, V132 is a whole-cell, heat-inactivated, *C. albicans* sublingual vaccine that has been designed as a novel therapy for RVVCs (22). The combination of MV140 with V132 in a single preparation primes human DCs to induce potent Th1/Th17 and Treg cells (22). Sublingual administration of MV140/V132 enhances proliferative responses of mice splenic $CD4^+$ T cells against related and unrelated antigens, suggesting trained immunity mechanisms (22). The specific contribution of each preparation to these effects and the underlying molecular mechanisms remains unknown. Herein, we show for the first time that sublingual vaccine *C. albicans* V132 induces trained immunity and enhances responses to MV140 both *in vitro* and *in vivo*. V132 induces metabolic and epigenetic rewiring characterized by a shift of cellular metabolism towards aerobic glycolysis and oxidative phosphorylation, enhancing transcription of pro-inflammatory cytokines. *In vivo*, sublingual administration of V132 in mice potentiates IL-17-mediated responses after *in vitro* stimulation with MV140. Our findings shed light into novel molecular mechanisms involved in the beneficial effects of these microbial sublingual preparations for GUTIs, which might well contribute to open new interventions with multiple clinical implications.

Materials and methods

Media and reagents

RPMI culture medium Dutch modified (Invitrogen) supplemented with 50 µg/mL gentamicin, 2 mM Glutamax (Gibco), and 1 mM pyruvate (Gibco) was used for human *in vitro* experiments. RPMI 1640 medium (Lonza) supplemented (cRPMI) with 10 % heat-inactivated fetal bovine serum (Gibco), 100 µg/mL normocin (*In vivo* Gen), 50 µg/mL penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mM sodium pyruvate (all from Life Technologies) was used for animal experiments. V132 composed of whole heat-inactivated *C. albicans*, MV140 (Uromune) composed of whole heat-inactivated bacteria (25% *E. coli*, 25% *P. vulgaris*, 25% *K. pneumoniae* and 25% *E. faecalis*), MV140/V132 (combination of MV140 and V132) and control excipients (glycerol, saline, pineapple flavor), identical for MV140 or V132, were provided by Inmunotek S.L. Lipopolysaccharide (LPS) from *E. coli* O55:B5 (Sigma-Aldrich) was used.

PBMC and monocyte isolation

Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMC) isolation was performed by using Ficoll-Paque density gradient media (GE Healthcare). Cells were washed twice in PBS, re-suspended in RPMI culture medium and counted. Monocyte isolation was performed by hyper-osmotic Percoll (Sigma) density gradient centrifugation (580*g*, 15 min). The interphase layer was collected, and cells were washed with cold PBS, re-suspended in RPMI culture medium Dutch modified and counted. An extra purification step was added by adhering Percoll-isolated monocytes or Ficoll-isolated PBMC to a flat-bottom 96-well plates for 1 h at 37°C. Subsequently, monocytes were washed with warm PBS to yield maximal purity.

In vitro trained immunity experiments

Trained immunity experiments were performed as previously described (42). 100 µL monocytes at 1 x 10^6 cells/mL or PBMC at 5 x 10^6 cells/mL density were added to a flat-bottom 96-well plates. Cells were incubated with control excipients (containing all excipients except the microbial components), V132 (3 FTU, Formazine Turbidity Units, per mL, as turbidity measurement for the monitoring of fungal suspensions), MV140 (10^7 bact. per mL) or MV140/V132 (MV140, 10^7 bact. per mL, and V132, 3 FTU per mL, as turbidity measurement for the monitoring of fungal suspensions) for 24 h. Supernatants from monocytes were collected 24 h after stimulation. Cells were washed once with 200 µL of warm PBS and incubated for 6 days in RPMI culture medium Dutch modified with 10% human pooled serum. On day 6, supernatants were collected for lactate measurement. Cells were re-stimulated for 24 h with 10 ng/mL LPS or 10^7 bact. per mL MV140 to assess the responsiveness of the cells to heterologous secondary stimulation, after which supernatants were collected.

Cytokine quantification

Cytokine production was quantified in supernatants using commercial ELISA kits for human IL-6, TNFα, IL-1β, IL-10, IL-1RA (R&D Systems); mouse IL-6, IFNγ, (BD Biosciences); TNFα, IL-10 (Invitrogen); and IL-17A (R&D Systems), following manufacturer's instructions.

Lactate measurement

Lactate concentration was measured from supernatants using a Lactate Fluorometric Assay Kit. Briefly, lactate was

oxidized resulting in H_2O_2 which was coupled to the conversion of Amplex Red reagent to fluorescent resorufin by horseradish peroxidase (HRP).

Viability assays

Cell viability was analyzed using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega), following manufacturer's recommendations. Briefly, released lactate dehydrogenase (LDH) upon cell lysis is measured with a 30-minute coupled enzymatic assay, which results in conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells.

Metabolic analysis

Adherent monocytes were obtained by incubating 5 x 10^7 PBMC in 10 cm Petri dishes for 1 h. The adherent monocytes were treated with 10 mL of RPMI culture medium Dutch modified containing control excipients, V132, MV140 or MV140/V132 for 24 h, washed once with warm PBS and incubated in RPMI culture medium Dutch modified with 10% human pooled serum at 37°C, 5% CO_2. Following 6 days in culture, cells were detached with Versene solution (ThermoFisher Scientific) and 5 x 10^5 cells were plated to overnight-calibrated cartridges in DMEM medium supplemented with 1 mM glutamine (pH adjusted to 7.4) and incubated for 1 h in a non-CO_2-corrected incubator at 37°C. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in response to 10 mM glucose, 1 µM oligomycin, 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 1.25 µM rotenone and 1.5 µM antimycin A injections were measured using a Seahorse XFe96 Analyzer (Agilent). A complete ECAR study was performed in two consecutive stages: basal extracellular acidification (after glucose injection), and mitochondrial complex V inhibition (oligomycin). A complete OCR study was performed in four consecutive stages: basal respiration (without drugs), mitochondrial complex V inhibition (oligomycin), maximal respiration induction (FCCP), and electron transportation chain inhibition (rotenone and antimycin A).

Chromatin immunoprecipitation

Adherent monocytes were obtained by incubating 5 x 10^7 PBMC in 10 cm Petri dishes for 1 h. The adherent monocytes were treated with 10 mL of RPMI culture medium Dutch modified containing control excipients, V132, MV140 or MV140/V132 for 24 h, washed once with warm PBS and incubated in RPMI culture medium Dutch modified with 10% human pooled serum at 37°C, 5% CO_2. Following 6 days in culture, cells were detached with Versene solution (ThermoFisher Scientific) and fixed in 1% methanol-free formaldehyde. Afterward, cells were sonicated using a Diagenode Bioruptor UCD-300 for 10 min (30 seconds on; 30 seconds off). 33 µL of the solution containing chromatin was incubated with 255 µL of dilution buffer, 12 µL of protease inhibitor cocktail and 1 µg of H3K27ac or H3K9me3 antibodies (Diagenode), overnight at 4°C with rotation. Protein A/G magnetic beads were washed in dilution buffer with 0.15% SDS and 0.1% BSA, added to the chromatin/antibody mix and rotated for 60 min at 4°C. Beads were washed with 500 µL buffer for 5 min at 4°C with five rounds of washes. After washing chromatin was eluted using elution buffer for 20 min. Supernatant was collected, 8 µL 5 M NaCl and 1 µL proteinase K were added, and samples were de-crosslinked at 64°C for 4 h. DNA was isolated with the MinElute PCR Purification Kit (QIAGEN), following manufacturer's protocol. Real-time quantitative PCR was performed using the SYBR Green method. Samples were analyzed by a comparative Ct method according to the manufacturer's instructions. The sequences of the used pair primers were: *IL6-1* (forward, TCGTGCATGACTTCAGCTTT; reverse GCGCTAAGAAGCAGAACCAC), *IL6-2* (forward, AGGGAGAGCCAGAACACAGA; reverse GAGTTT CCTCTGACTCCATCG), *TNFA1* (forward, AGAGGA CCAGCTAAGAGGGA; reverse AGCTTGTCAGGGGAT GTGG), and *TNFA2* (forward, GTGCTTGTTCCTCAGCCTCT; reverse ATCACTCCAAAGTGCAGCAG).

Mice experiments

All mice procedures included in this study were reviewed and ethically approved by Universidad Complutense de Madrid (UCM) and Comunidad Autónoma de Madrid (CAM) within the context of project SAF-2017-84978-R, (CAM: ref.10/250312.9/18). BALB/c mice (female, 6 weeks old, Charles River) were intraperitoneally immunized with 20 µL of control excipients or V132 (300 FTU per mL) at day 0 and 3 and sacrificed 4 days after the last immunization. In another animal model, BALB/c mice (6 weeks old) were sublingually immunized for 5 consecutive days during 2 weeks with 20 µL of control excipients or V132 (300 FTU per mL) and sacrificed 3 days after last immunization. Moreover, some immunized mice were sublingually administered with 20 µL of MV140 (10^9 bact. per mL) 3 days after last sensitization and sacrificed after 8 days of the MV140 administration. Peritoneal cells and spleens were collected.

Peritoneal lavage and peritoneal cell culture

Peritoneal cells were collected with PBS/EDTA 2mM and resuspended in cRPMI. Duplicates of 0.25 x 10^6 peritoneal cells

were cultured *in vitro* with control excipients or MV140 (10^6 bact. per mL) in flat-bottom 96-well plates for 24 h at 37°C and 5% CO_2. Cell-free supernatants of duplicates were pooled and used to quantify IL-6 and TNFα.

Spleen processing and splenocyte cell culture

Spleens were minced and filtered through 40 µm nylon cell strainers to obtain a single-cell suspension. Then, red blood cells were lysed with ACK lysis buffer before being resuspended in cRPMI. Splenocyte viability was assessed using Trypan Blue exclusion. Triplicates of 0.8 x 10^6 splenocytes were cultured *in vitro* with control excipients or MV140 (10^5-10^7 bact. per mL) in flat-bottom 96-well plates. After 24 or 72 h of culture at 37°C and 5% CO_2, the triplicates were pooled and cell-free supernatants were used to quantify IL-6, TNFα, IL-17, IFNγ and IL-10 by ELISA.

Statistics

All the data are expressed as means ± s.e.m. of the indicated parameters. One-way ANOVA, Wilcoxon test or Unpaired Student *t* test for statistical analysis were performed using GraphPad Prism software, version 6.0. Significance was defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Results

Exposure of human peripheral blood mononuclear cells to V132 enhances pro-inflammatory cytokine production after secondary stimulation with LPS

To assess whether priming of human peripheral blood mononuclear cells (PBMC) with V132, MV140, or a simultaneous combination of V132 and MV140 would increase PBMC's responses upon second stimulation, an *in vitro* model for the induction of trained immunity was employed (43, 44). As shown in Figure 1A, PBMC were initially treated with control excipients (ctrl), V132 alone, MV140 alone, and the combination of MV140 and V132 (MV140/V132) and the cytokine signature after 24 h of stimulation quantified. Next, cells were washed, left resting for 6 days with medium, and then re-stimulated with LPS to assess cytokine production after 24 h (Figure 1A). PBMC stimulation with V132 alone induced significant IL-6 and anti-inflammatory IL-1RA production (Figure 1B). Exposure to MV140 and MV140/V132 significantly increased the production of IL-6, TNFα and IL-1β compared to control excipients or V132 (Figure 1B), without affecting cell viability (Supplementary Figure 1A). PBMC stimulated with MV140 alone produced significantly increased IL-1β compared to the MV140/V132 combination (Figure 1B). Although overall IL-10 production was higher after stimulation with MV140 than with control,

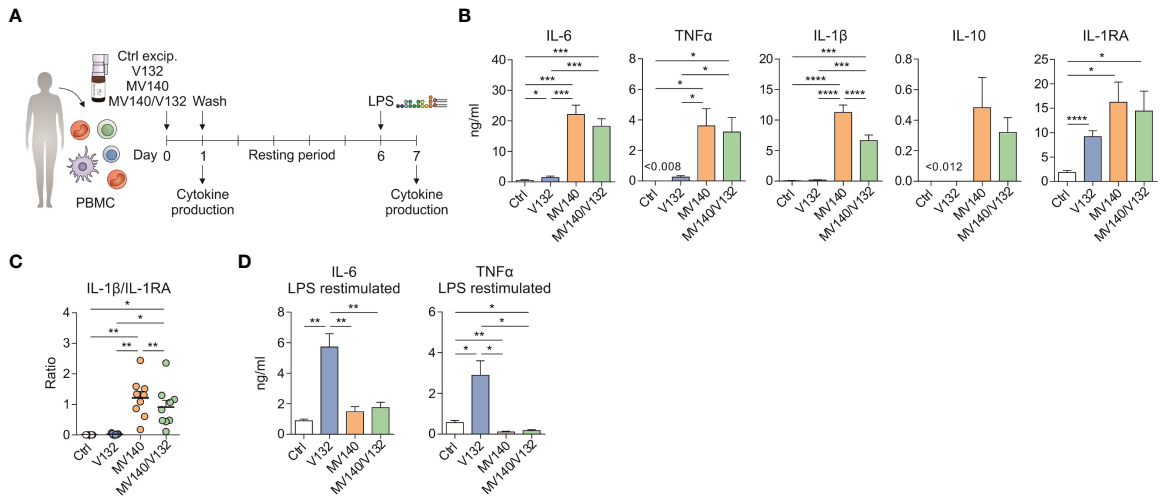

FIGURE 1
V132 induces trained immunity in human PBMC. **(A)** Human *in vitro* model for the induction of trained immunity in human PBMC. **(B)** Cytokine production after stimulation of human PMBC with control excipients (ctrl), V132, MV140 or MV140/V132 for 24 h in cell-free supernatants (n = 6-11 donors of two or four independent experiments). **(C)** IL-1β/IL-1RA ratio produced by human PBMC after 24 h in the indicated conditions (n = 9 donors of four independent experiments). **(D)** PBMC were incubated for 24 h with ctrl, V132, MV140 or MV140/V132. After 6 days, they were re-stimulated with LPS and cytokines were measured 24 h later (n = 11 donors of four independent experiments). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

V132 or MV140/V132, significant differences were not detected (Figure 1B). MV140 or MV140/V132-stimulated PBMC also significantly produced higher IL-1RA levels after 24 h, with higher levels observed after MV140 or MV140/V132 than V132 stimulation (Figure 1B). Consequently, the IL-1β/IL-1RA ratio was significantly higher when PBMC were stimulated with MV140 or MV140/V132 than control excipients or V132, and slight but higher with MV140 than with MV140/V132 (Figure 1C). PBMC conditioned with V132 displayed a trained response on day 7 after re-stimulation with LPS resulting in a significant IL-6 and TNFα production than control excipients (Figure 1D). This was not the case when PBMC were stimulated with MV140, and remarkably not with V132 in the presence of MV140 (MV140/V132) (Figure 1D). No IL-10 production was observed on day 7 in these cell cultures (data not shown). Collectively, these results suggest that in the assayed conditions, V132 but not MV140 or MV140/V132 induced trained immunity in human PBMC (Figure 1D).

Exposure of human PBMC to V132 induces metabolic and epigenetic reprogramming

Trained cells undergo strong metabolic and epigenetic reprogramming, two major molecular mechanisms underlying trained immunity (26). Therefore, we first studied the metabolic changes imprinted by V132 in PBMCs compared to those induced by control excipients, MV140 or MV140/V132. PBMC treated with V132, MV140 or MV140/V132 produced significantly higher lactate concentrations in cell culture supernatants after 6 days of resting (prior to re-stimulation) compared to control cultures with excipients, reflecting an increase in the activity of glycolysis (Figure 2A). Remarkably, the levels of lactate produced by V132-trained PBMC were significantly higher than MV140- or MV140/V132-treated cells (Figure 2A). To further verify these results, we performed functional metabolic experiments using a Seahorse bioanalyzer to monitor real-time extracellular

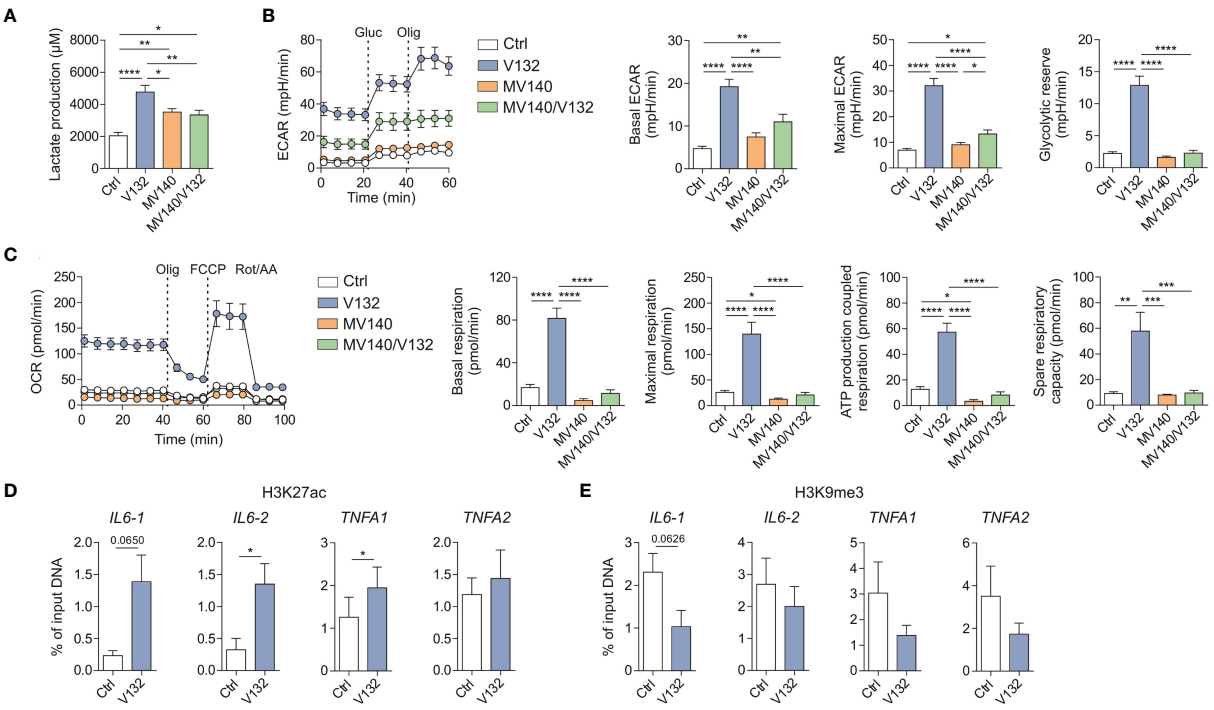

FIGURE 2
V132-trained cells exhibit metabolic and epigenetic rewiring. Human PBMC were incubated for 24 h with control excipients (ctrl), V132, MV140 or MV140/V132. On day 6 (prior to restimulation), metabolic and epigenetic assays were performed. **(A)** Lactate production by human PBMC was quantified (n = 12 donors of four independent experiments). **(B)** Kinetic study of extracellular acidification rate (ECAR) in ctrl-, V132-, MV140-, or MV140/V132-trained PBMC by sequential addition of glucose (Gluc) and oligomycin (Olig) (n = 7 donors of three independent experiments, 5 repeated measures of each donor). **(C)** Kinetic study of mitochondrial oxygen consumption rate (OCR) in ctrl-, V132-, MV140-, or MV140/V132-trained PBMC by sequential addition of oligomycin (Olig), (FCCP) and rotenone/antimycin A (Rot/AA) (n = 7 donors of three independent experiments, 5 repeated measures of each donor). H3K27ac **(D)** and H3K9me3 **(E)** histone modifications were determined at the promoter sites of *IL6* and *TNFA* using two different primers for each one (n = 5-6 donors of two independent experiments). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA **(A-C)** or Paired Student *t* test **(D, E)**. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

acidification rate (ECAR) and mitochondrial oxygen consumption rate (OCR) after 6 days of resting of the initially exposed PBMC. V132-trained PBMC showed an increased basal and maximal ECAR, as well as glycolytic reserve compared to PBMC stimulated with MV140, MV140/V132 or control excipients (Figure 2B). Only a slight but significant increase in basal and maximal ECAR was observed after the treatment with MV140/V132 compared to MV140 or control excipients (Figure 2B). In addition, basal and maximal respiration, ATP production coupled to respiration, and spare respiratory capacity were significantly higher after V132 training than the other assayed stimuli, also supporting the induction of mitochondrial oxidative phosphorylation in V132-trained PBMC (Figure 2C).

To determine whether the enhanced production of pro-inflammatory cytokines after the second re-stimulation with LPS might be linked to V132-induced epigenetic changes, we studied chromatin status near the *IL6* and *TNFA* genes by quantifying H3K27ac and H3K9me3 histone marks by chromatin immunoprecipitation (ChIP) analysis. H3K27ac is previously described as the histone mark associated with active chromatin and increased gene transcription in trained immunity (45). In contrast, H3K9me3 is considered a repressor mark (46). V132-trained PBMC display an increase of H3K27ac and a decrease of H3K9me3 at the promoters of *IL6* and *TNFA* (Figure 2D, E, respectively). Collectively, these results demonstrate that the induction of trained immunity with V132 in PBMC is mediated by metabolic and epigenetic reprogramming.

V132 induces trained immunity in purified human monocytes

To confirm that V132 induces trained in purified human monocytes, the same *in vitro* trained immunity protocol described above was performed using human monocytes isolated from healthy donors (Figure 3A). Initial exposure of monocytes to V132 alone did not induce pro-inflammatory cytokines, in contrast to the IL-6, IL-1β and TNFα production when stimulated with MV140 or MV140/V132 (Figure 3B). Cell viability was not affected in these cultures (Supplementary Figure 1B). Interestingly, monocytes simultaneously activated with MV140/V132 displayed significantly higher levels of TNFα and lower IL-1β than MV140 alone (Figure 3B). Stimulation of monocytes with the indicated conditions increased anti-inflammatory cytokine IL-1RA production, which was significantly higher upon V132 treatment than MV140 or MV140/V132 (Figure 3B), without IL-10 production (data not shown). The IL-1β/IL-1RA ratio was significantly higher when monocytes were treated with MV140 or MV140/V132 than with V132 (Figure 3C). MV140-treated monocytes showed a higher IL-1β/IL-1RA ratio than cells treated with MV140/V132

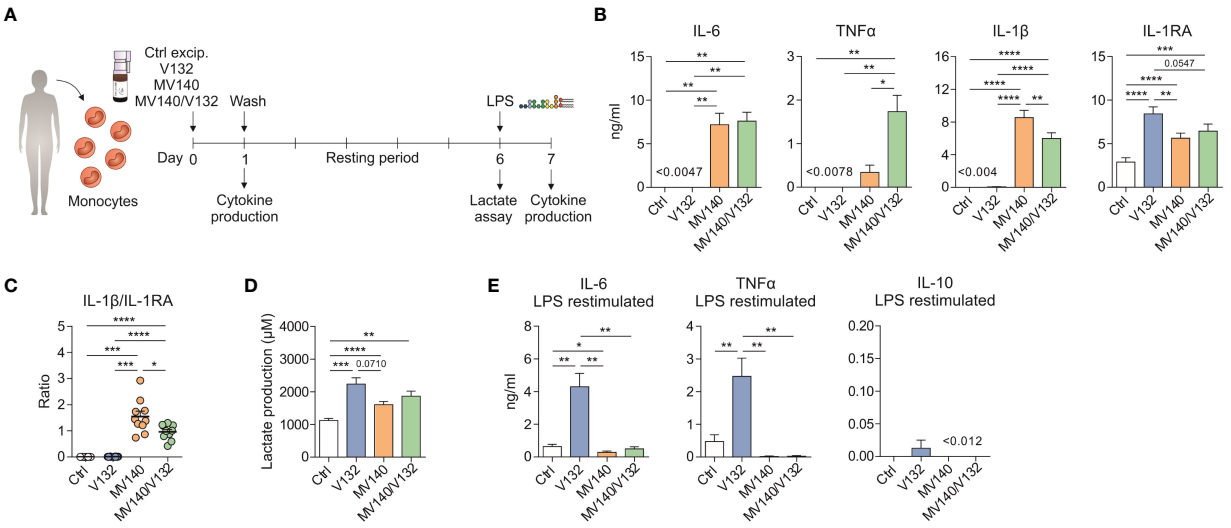

FIGURE 3
V132 induces trained immunity in human monocytes. **(A)** Human *in vitro* model for the induction of trained immunity in human monocytes. **(B)** Cytokine production after stimulation of human monocytes with control excipients (ctrl), V132, MV140 or MV140/V132 for 24 h in cell-free supernatants (n = 10 donors of four independent experiments). **(C)** IL-1β/IL-1RA ratio produced by human monocytes after 24 h in the indicated conditions (n = 10 donors of four independent experiments). **(D)** Lactate production by human monocytes 6 days after 24 h stimulation with ctrl, V132, MV140 or MV140/V132, followed by rest in culture media (n = 11 donors of four independent experiments). **(E)** Monocytes were incubated for 24 h with ctrl, V132, MV140 or MV140/V132. On day 7, after 24 h restimulation with LPS, IL-6, TNFα and IL-10 concentration in the supernatants were measured (n = 10 donors of four independent experiments). Values are mean ± SEM. Statistical significance was determined using One-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

10.3389/fimmu.2022.1066383

(Figure 3C). Monocytes treated with V132, MV140 or MV140/V132 significantly increased the production of lactate after 6 days of resting, reflecting an increased glycolysis metabolism (Figure 3D). Supporting our previous data with PBMC, monocytes trained with V132 produced higher amounts of lactate compared to the other assayed stimuli, without significant differences observed in this case (Figure 3D). V132-trained monocytes produced significantly higher amounts of IL-6 and TNFα upon re-stimulation 7 days after first priming and resting with LPS, without IL-10 induction (Figure 3E). Collectively, these data indicate that in the assayed conditions V132 but not MV140 or MV140/V132 induced trained immunity also in purified human monocytes.

V132-trained human monocytes display enhanced innate pro-inflammatory responses upon secondary stimulation with MV140

Next, we wanted to assess whether V132-trained monocytes could display an enhanced response to MV140 used as secondary stimulus (Figure 4A). V132-trained monocytes treated with MV140 produced significantly higher concentration of IL-6 and TNFα compared to control monocytes (Figure 4B). The production of pro-inflammatory cytokines by V132-trained monocytes was higher upon secondary stimulation with MV140 than LPS (Figure 4B).

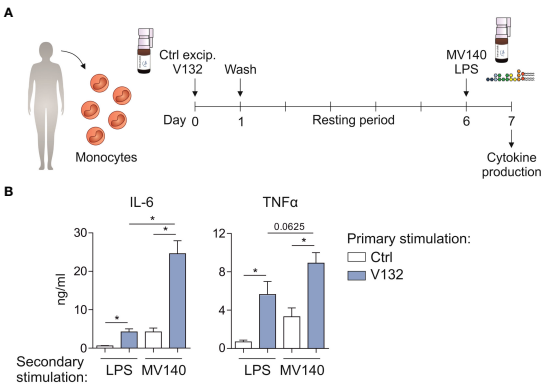

FIGURE 4
V132-trained monocytes display enhanced innate immune responses upon *in vitro* secondary treatment with MV140. **(A)** Human *in vitro* model for the induction of trained immunity in monocytes. **(B)** Monocytes were incubated for 24 h with control excipients (ctrl) or V132 (primary stimulation). On day 7, after 24 h restimulation with LPS or MV140 (secondary stimulation), IL-6 and TNFα concentration in the supernatants were measured (n = 6 donors of two independent experiments). Values are mean ± SEM. Statistical significance was determined using Wilcoxon test. *$P < 0.05$.

Collectively, these data indicate that exposure of monocytes to V132 significantly enhances their capacity to mount potent pro-inflammatory responses upon exposure to MV140.

V132 induces *in vivo* trained immunity in mice and enhances Th17 responses triggered by MV140

To assess the *in vivo* relevance of V132-induced trained immunity, BALB/c mice were immunized by intraperitoneally administration of control excipients or V132 as shown in Figure 5A. Intraperitoneal immunization with V132 significantly increased the accumulation of infiltrating cells into the peritoneal cavity compared to controls (Figure 5B). Peritoneal cells and splenocytes from both V132- and control mice did respond to MV140 *in vitro* by producing IL-6 and TNFα (Figure 5C, D, respectively). However, cells derived from V132-immunized mice produced significantly higher levels of IL-6 and TNFα than those from control mice (Figure 5C, D). To further confirm the results when V132 was administered through the sublingual route, BALB/c mice were immunized as shown in Figure 5E. Splenocytes from both V132-immunized and control mice produced IL-6 and TNFα *in vitro* upon MV140 stimulation (Figure 5F). Interestingly, the production of TNFα, but not of IL-6, were higher in mice immunized sublingually with V132 (Figure 5F).

To assess potential effects of V132-induced training *in vivo* on adaptive immune responses, BALB/c mice immunized sublingually with control excipients or V132 were treated *in vivo* with MV140 as shown in Figure 6A. Sublingual immunization with V132 and subsequent sublingual treatment with MV140 significantly increased the cell infiltration into the peritoneal cavity (Figure 6B). Peritoneal cells from both mice groups activated *in vitro* with MV140 produced significantly higher concentrations of IL-6 and TNFα than those without MV140 stimulation (Figure 6C). The levels of TNFα, but not of IL-6, produced by peritoneal cells from V132-sublingually immunized mice were slightly and significantly higher than those induced by peritoneal cells from control mice after *in vitro* stimulation with MV140 (Figure 6C). To assess whether V132 enhances specific T cell responses to MV140, splenocytes from immunized mice (Figure 6A) were stimulated *in vitro* with or without MV140 (Figure 6D). As shown, mice both immunized sublingually with V132 or control mice produced a significant IL-17A, IFNγ and IL-10 *in vitro* response to MV140 stimulation (Figure 6D). Remarkably, the levels of IL-17A, but not IFNγ or IL-10, produced by splenocytes from V132-immunized mice after *in vitro* MV140 stimulation were significantly higher than those induced by splenocytes from control mice (Figure 6D), indicating that sublingual V132 vaccination potentiates Th17 adaptive immune responses in mice against MV140.

Discussion

In this study, we demonstrate that V132, a heat-inactivated, whole-cell *Candida albicans* sublingual vaccine, induces trained immunity in human PBMC and purified monocytes, which in turn significantly enhances immune responses triggered by the MV140 sublingual vaccine, a whole-cell, heat-inactivated, polybacterial formulation. V132-induced trained immunity is mediated by the induction of metabolic reprogramming mirrored by increased glycolysis and lactate production as well as enhanced oxidative phosphorylation. In addition, V132 induces epigenetic reprogramming at the level of H3K27ac and H3K9me3 within the promoter regions of *IL6* and *TNFA* genes, which results in enhanced transcription of these pro-inflammatory genes upon subsequent exposition to MV140. Supporting these findings, intraperitoneal or sublingual *in vivo* immunization of mice with V132 generates peritoneal cells and splenocytes able to display enhanced innate immune responses upon *in vitro* stimulation with MV140. Remarkably, splenocytes from V132-sublingually immunized mice and treated *in vivo* with MV140 show a significant increase of Th17 responses as detected by *in vitro* stimulation with MV140. Overall, we shed light into unprecedented molecular mechanisms by which V132 might contribute to enhance innate and adaptive immune responses triggered by MV140, which might help to pave the way for future novel interventions for GUTIs.

GUTIs, including RUTIs and RVVCs, are among the most prevalent bacterial and fungal infections worldwide, requiring repeated administrations of antibiotics and antifungals (2, 47). Long-term use of antibiotics and antifungals are associated with the development of drug resistances and microbiota alterations. Alternative strategies able to overcome such deleterious effects while preserving efficacy are highly needed (1, 13). In this regard, different approaches have been considered for RUTIs (21, 48, 49) and RVVCs (50–52). Among these alternatives, several studies have focused on the development of TIbV over the last years (19, 24, 53). TIbV induce innate immune memory, thus conferring heterologous protection against a broad range of pathogens (19). The use of TIbV might also offer great potential for infections without or with no effective treatments available, for pathogens with high mutation rates as well as for diseases in which co-infections takes place such as GUTIs (19). The sublingual formulation MV140 prevents RUTIs in up to 80-90% of patients (34–41). RUTIs are also frequently associated with RVVCs, with the V132 *C. albicans* formulation being developed as a potential alternative to antifungals (22). We previously showed that the use of MV140/V132 in a single sublingual preparation enhanced IgG and IgA responses in mice

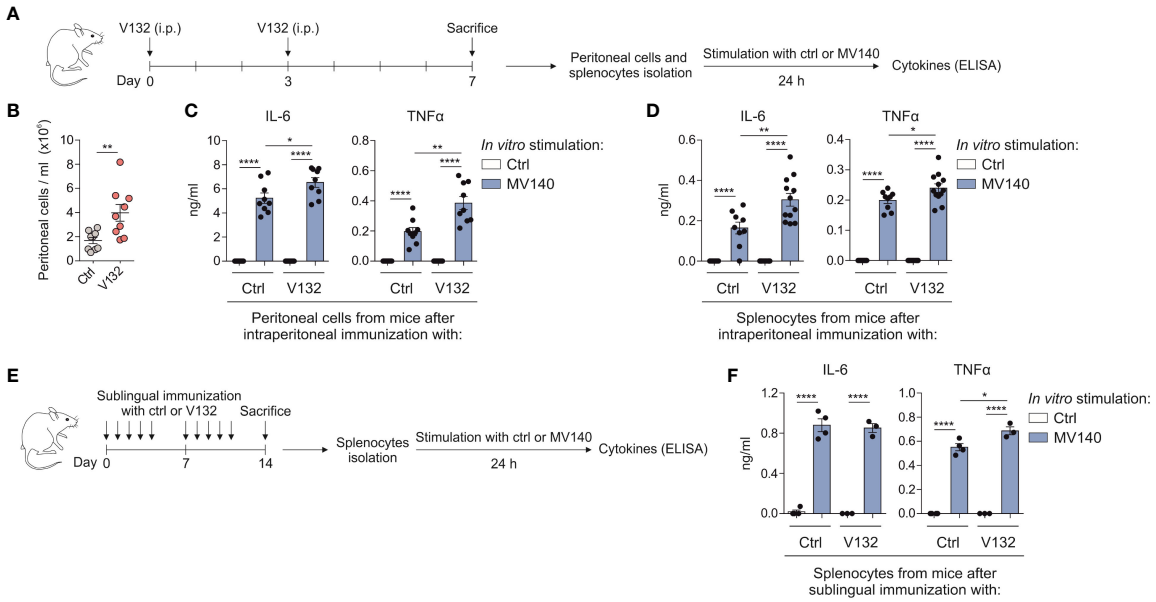

FIGURE 5
V132 induces trained immunity in mice and enhances innate immune responses after MV140 *in vitro* stimulation. **(A)** Scheme of the intraperitoneal immunization protocol and analysis of induced systemic response; i.p., intraperitoneal; ctrl; control excipients. **(B)** Concentration of peritoneal cells in the peritoneal cavity 4 days after last intraperitoneal immunization (n = 9 mice of two independent experiments). **(C)** Cytokine production by peritoneal cells from the indicated mice, stimulated *in vitro* with ctrl or MV140 for 24 h (n = 9 mice of two independent experiments). **(D)** Cytokine production by splenocytes from the indicated mice, stimulated *in vitro* with ctrl or MV140 for 24 h (n = 9-12 mice of two independent experiments). **(E)** Scheme of the sublingual immunization protocol and analysis of induced systemic response; ctrl; control excipients. **(F)** Cytokine production by splenocytes from the indicated mice, stimulated *in vitro* with ctrl or MV140 for 24 h (n = 3-4 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired *t* test. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

against all the antigens contained in the vaccine preparation (22). In addition, MV140/V132 imprinted human DCs with the capacity to prime potent adaptive immune responses against both related and unrelated antigens (22). However, whether V132 could induce trained immunity and how this could contribute to modulate the innate and adaptive immune responses induced by MV140 remained elusive until this work.

Here we show that V132-induced trained immunity in human PBMC and monocytes significantly enhanced innate and adaptive immune responses triggered by MV140. β-glucan, the cell wall component of *C. albicans* is, together with live attenuated *Mycobacterium bovis* BCG vaccine, one of the best characterized trained immunity inducers (30, 43, 44). First stimulation of monocytes with V132 did not induce production of pro-inflammatory cytokines after 24h (43), and only the inhibitory cytokine IL-1RA was observed, as previously described for other trained immunity inducers (54, 55). The binding of IL-1RA to the same receptor as IL-1β, inhibits IL-1β-induced signaling and controlling inflammation during infections (56). *In vitro*, V132 training increases IL-6 and TNFα production after second stimulation with LPS or MV140.

To confirm the relevance of our findings, mice were immunized with V132 to assess its capacity to induce trained immunity *in vivo*. V132 *in vivo* immunization significantly enhanced immune responses when peritoneal cells and splenocytes were stimulated *in vitro* with MV140. Previously, studies reported that TIbV might also potentiate adaptive responses against different pathogens (19, 20, 57–59). Our *in vivo* results showed that the sublingual immunization of V132 and a subsequent stimulation with MV140 in mice promotes specific systemic Th17 responses, suggesting the potential immunomodulatory capacity of V132 to enhance adaptive immune responses. In contrast to V132, when human monocytes were primed *in vitro* with MV140 or MV140/V132 a significant increase of concentrations of pro-inflammatory cytokines and IL-1RA was detected after 24 h. However, after 6 days of resting, IL-6 and TNFα production upon second stimulation of monocytes with LPS were very low, suggesting that MV140 or MV140/V132 generate unresponsive monocytes to the secondary LPS exposition. Supporting these results, it has been previously described that TLR signaling through LPS may counteract the induction of trained immunity by *C. albicans* cell wall component β-glucan (58) and that LPS promotes tolerance in human monocytes, keeping the cells in an unresponsive state to secondary stimulation (33, 45). It is important to keep in mind that MV140 is composed of 75% of Gram-negative bacteria, thus containing LPS that might also partially reverse the V132-induced trained immunity. The main aim of including MV140 together V132 in the vaccine formulation is to incorporate the bacterial antigens contained in MV140 preparation to induce adaptive immune responses against all the stimuli.

Metabolic and epigenetic reprogramming are hallmarks underlying trained immunity (26, 29). Trained cells use different metabolic pathways to adapt their function for the production of energy faster and more efficiently (30–32). β-glucan-trained cells displayed a high glycolysis rate and Warburg effect but decreased oxidative phosphorylation, depending on Akt/mTOR/HIF-1a pathway (30, 31). We have previously reported that V132 activates the Akt/mTOR signaling pathway in human DCs (22). Here, we showed that V132-induced trained immunity enhances not only glycolysis with a higher lactate production, but also oxygen consumption. Arts et al. reported that BCG training also induced both glycolysis and oxidative phosphorylation in human

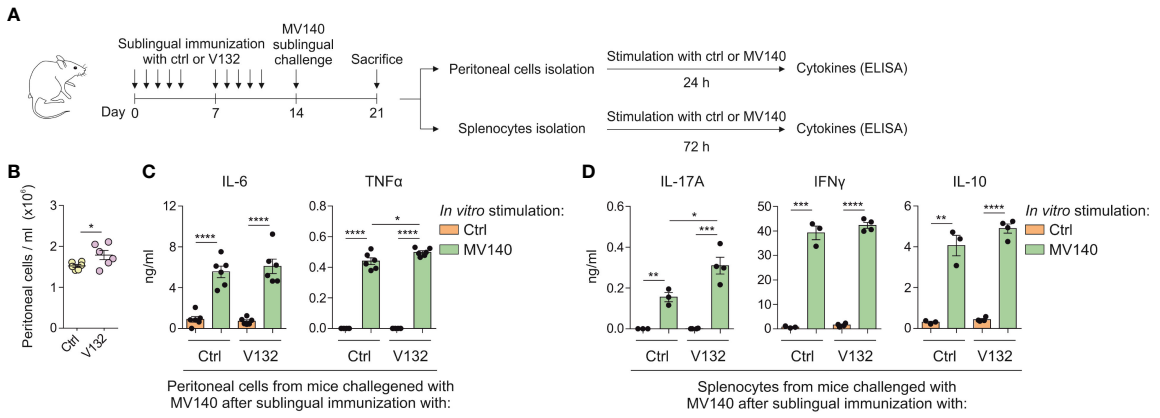

FIGURE 6
V132 induces trained immunity in mice and enhances adaptive immune responses against MV140. **(A)** Scheme of the sublingual immunization protocol and analysis of induced systemic response; ctrl; control excipients. **(B)** Concentration of peritoneal cells in the peritoneal cavity 7 days after stimulation with MV140 (n = 6 mice of one independent experiment). **(C)** Cytokine production by peritoneal cells from the indicated mice, stimulated *in vitro* with ctrl or MV140 for 24 h (n = 6 mice of one independent experiments). **(D)** Cytokine production by splenocytes from the indicated mice, stimulated *in vitro* with ctrl or MV140 for 72 h (n = 3-4 mice of one independent experiment). Values are mean ± SEM. Statistical significance was determined using Unpaired *t* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

monocytes, suggesting that different metabolic pathways can be activated depending on the training stimuli (32). Metabolic rewiring directly contributes to epigenetic reprogramming of trained monocytes (27, 32). Training of monocytes promotes epigenetic changes at promoters of genes of pro-inflammatory cytokines (27). Our chromatin immunoprecipitation experiments showed that V132 training results in an increased H3K27ac and a decreased H3K9me3 at promoter regions of *IL6* and *TNFA* genes, as previously showed for other trained immunity inducers (20, 32, 43, 60, 61).

The use of *C. albicans* as trained immunity inducer is well described, as well as its cell wall component β-glucan (43). Here, we have studied the V132 (*C. albicans*) used in a sublingual vaccine under clinical development for the prevention of recurrent vulvovaginal candidiasis. This fungal infection is associated with the use of antibiotics (6), as those indicated for treating recurrent urinary tract infections, and therefore in a number of instances both types of infections coexist (4). In this context, prior vaccination with V132 as a trained immunity inducer may be useful to potentiate MV140, a sublingual vaccine indicated for the prevention of recurrent urinary tract infections (41). It should be noted that mucosal tissues are excellent targets for inducing trained immunity by whole cell microorganisms (62, 63), supporting the approach we describe here. On the other hand, vaccination through the sublingual route induces strong immune responses in local and peripheral lymphoid organs as well as in distant mucosa such as the genitourinary tract (64–66).

In summary, we provide novel insights into the mechanisms through which V132-induced trained immunity might enhance both innate and adaptive immune responses triggered by MV140. An improved understanding of the underlying mechanisms of sublingual fungal and bacterial vaccines may well contribute to develop novel therapeutic strategies for GUTIs and other immune-mediated diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Nijmegen, the Netherlands). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. All mice procedures included in this study were reviewed and ethically approved by Universidad Complutense de Madrid (UCM) and Comunidad Autónoma de Madrid (CAM) within the context of project SAF-2017-84978-R, (CAM:ref.10/250312.9/18).

Author contributions

Conceived and designed the study: OP and JD-A. Performed the experiments: LM-C (human and mice experiments), AA (mice experiments), IB and ÖB (technical support for human experiments). Provided reagents: JS, MN, JD-A, and OP. Analyzed and discussed the data: LM-C, AA, IB, ÖB, JS, MN, JD-A, and OP. Wrote the paper: LM-C and OP. All the authors revised the manuscript, contributed with revisions and approved the final version of the manuscript.

Funding

The authors laboratories are supported by grant PID2020-114396RB-I00 to OP from MINECO, Spain, by unrestricted grant from Inmunotek under an Art.83 UCM contract to OP; The Netherlands Organization for Scientific Research (VENI grant 09150161910024 and Off Road grant 04510012010022) to JD-A; ERC Advanced Grant (#833247) and a Spinoza Grant of the Netherlands Organization for Scientific Research to MGN. LM-C is a recipient of FPU predoctoral fellowships from MINECO, Spain.

Conflict of interest

OP has received fee for lectures or participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, Diater, GSK, Pfizer, Inmunotek SL, Novartis, Sanofi Genzyme, Stallergenes and Regeneron. OP has received research grants from Inmunotek SL, Novartis SL, MINECO, MICINNIN and CAM. JS is the founder and CEO of Inmunotek SL.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1066383/full#supplementary-material

SUPPLEMENTARY FIGURE 1

(A) Cytotoxicity assay after stimulation of human PBMC with control excipients (ctrl), V132, MV140 or MV140/V132 for 24 h. Lysed cells are included as a control (n = 6 donors of two independent experiments). **(B)** Cytotoxicity assay after stimulation of human monocytes with ctrl, V132, MV140 or MV140/V132 for 24 h. Lysed cells are included as a control (n = 6 donors of two independent experiments). Values are mean ± SEM.

References

1. Klein RD, Hultgren SJ. Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. *Nat Rev Microbiol* (2020) 18 (4):211–26. doi: 10.1038/s41579-020-0324-0

2. Foxman B. Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. *Infect Dis Clin North Am* (2014) 28(1):1–13. doi: 10.1016/j.idc.2013.09.003

3. Bixler BR, Anger JT. Updates to recurrent uncomplicated urinary tract infections in women: AUA/CUA/SUFU guideline. *J Urol* 2022:101097JU0000000000002888. doi: 10.1097/JU.0000000000002888

4. Butler AM, Durkin MJ, Keller MR, Ma Y, Powderly WG, Olsen MA. Association of adverse events with antibiotic treatment for urinary tract infection. *Clin Infect Dis* (2022) 74(8):1408–18. doi: 10.1093/cid/ciab637

5. Wawrysiuk S, Naber K, Rechberger T, Miotla P. Prevention and treatment of uncomplicated lower urinary tract infections in the era of increasing antimicrobial resistance-non-antibiotic approaches: a systemic review. *Arch Gynecol Obstet* (2019) 300(4):821–8. doi: 10.1007/s00404-019-05256-z

6. Shukla A, Sobel JD. Vulvovaginitis caused by candida species following antibiotic exposure. *Curr Infect Dis Rep* (2019) 21(11):44. doi: 10.1007/s11908-019-0700-y

7. Rosati D, Bruno M, Jaeger M, Ten Oever J, Netea MG. Recurrent vulvovaginal candidiasis: An immunological perspective. *Microorganisms* (2020) 8(2):144. doi: 10.3390/microorganisms8020144

8. Sobel JD. Vulvovaginal candidosis. *Lancet* (2007) 369(9577):1961–71. doi: 10.1016/S0140-6736(07)60917-9

9. Sobel JD. Recurrent vulvovaginal candidiasis. *Am J Obstet Gynecol* (2016) 214 (1):15–21. doi: 10.1016/j.ajog.2015.06.067

10. Denning DW, Kneale M, Sobel JD, Rautemaa-Richardson R. Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* (2018) 18(11):e339–e47. doi: 10.1016/S1473-3099(18)30103-8

11. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Clinical practice guideline for the management of candidiasis: 2016 update by the infectious diseases society of America. *Clin Infect Dis* (2016) 62 (4):e1–50. doi: 10.1093/cid/civ933

12. Yano J, Sobel JD, Nyirjesy P, Sobel R, Williams VL, Yu Q, et al. And Fidel PL, jr. current patient perspectives of vulvovaginal candidiasis: incidence, symptoms, management and post-treatment outcomes. *BMC Womens Health* (2019) 19(1):48. doi: 10.1186/s12905-019-0748-8

13. Fisher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, et al. Tackling the emerging threat of antifungal resistance to human health. *Nat Rev Microbiol* (2022) 20(9):557–71. doi: 10.1038/s41579-022-00720-1

14. Marchaim D, Lemanek L, Bheemreddy S, Kaye KS, Sobel JD. Fluconazole-resistant candida albicans vulvovaginitis. *Obstet Gynecol* (2012) 120(6):1407–14. doi: 10.1097/AOG.0b013e31827307b2

15. Micoli F, Bagnoli F, Rappuoli R, Serruto D. The role of vaccines in combatting antimicrobial resistance. *Nat Rev Microbiol* (2021) 19(5):287–302. doi: 10.1038/s41579-020-00506-3

16. Tejera-Alhambra M, Palomares O, Perez de Diego R, Diaz-Lezcano I, Sanchez-Ramon S. New biological insights in the immunomodulatory effects of mucosal polybacterial vaccines in clinical practice. *Curr Pharm Des* (2016) 22 (41):6283–93. doi: 10.2174/1381612822666160829143129

17. Prattley S, Geraghty R, Moore M, Somani BK. Role of vaccines for recurrent urinary tract infections: A systematic review. *Eur Urol Focus* (2020) 6(3):593–604. doi: 10.1016/j.euf.2019.11.002

18. Oliveira LVN, Wang R, Specht CA, Levitz SM. Vaccines for human fungal diseases: close but still a long way to go. *NPJ Vaccines* (2021) 6(1):33. doi: 10.1038/s41541-021-00294-8

19. Sanchez-Ramon S, Conejero L, Netea MG, Sancho D, Palomares O, Subiza JL. Trained immunity-based vaccines: A new paradigm for the development of broad-spectrum anti-infectious formulations. *Front Immunol* (2018) 9:2936. doi: 10.3389/fimmu.2018.02936

20. Kleinnijenhuis J, Quintin J, Preijers F, Benn CS, Joosten LA, Jacobs C, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J Innate Immun* (2014) 6(2):152–8. doi: 10.1159/000355628

21. Benito-Villalvilla C, Cirauqui C, Diez-Rivero CM, Casanovas M, Subiza JL, Palomares O. MV140, a sublingual polyvalent bacterial preparation to treat recurrent urinary tract infections, licenses human dendritic cells for generating Th1, Th17, and IL-10 responses *via* syk and MyD88. *Mucosal Immunol* (2017) 10 (4):924–35. doi: 10.1038/mi.2016.112

22. Martin-Cruz L, Sevilla-Ortega C, Benito-Villalvilla C, Diez-Rivero CM, Sanchez-Ramon S, Subiza JL. And palomares o. a combination of polybacterial MV140 and candida albicans V132 as a potential novel trained immunity-based vaccine for genitourinary tract infections. *Front Immunol* (2020) 11:612269. doi: 10.3389/fimmu.2020.612269

23. Cirauqui C, Benito-Villalvilla C, Sanchez-Ramon S, Sirvent S, Diez-Rivero CM, Conejero L, et al. Human dendritic cells activated with MV130 induce Th1, Th17 and IL-10 responses *via* RIPK2 and MyD88 signalling pathways. *Eur J Immunol* (2018) 48(1):180–93. doi: 10.1002/eji.201747024

24. de Bree LCJ, Koeken V, Joosten LAB, Aaby P, Benn CS, van Crevel R, et al. Non-specific effects of vaccines: Current evidence and potential implications. *Semin Immunol* (2018) 39:35–43. doi: 10.1016/j.smim.2018.06.002

25. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe* (2011) 9(5):355–61. doi: 10.1016/j.chom.2011.04.006

26. Dominguez-Andres J, Netea MG. Long-term reprogramming of the innate immune system. *J Leukoc Biol* (2019) 105(2):329–38. doi: 10.1002/JLB.MR0318-104R

27. Netea MG, Dominguez-Andres J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. *Nat Rev Immunol* (2020) 20(6):375–88. doi: 10.1038/s41577-020-0285-6

28. O'Neill LA, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nat Rev Immunol* (2016) 16(9):553–65. doi: 10.1038/nri.2016.70

29. Fanucchi S, Dominguez-Andres J, Joosten LAB, Netea MG, Mhlanga MM. The intersection of epigenetics and metabolism in trained immunity. *Immunity* (2021) 54(1):32–43. doi: 10.1016/j.immuni.2020.10.011

30. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* (2014) 345(6204):1250684. doi: 10.1126/science.1250684

31. Arts RJ, Novakovic B, Ter Horst R, Carvalho A, Bekkering S, Lachmandas E, et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenetic programs in trained immunity. *Cell Metab* (2016) 24(6):807–19. doi: 10.1016/j.cmet.2016.10.008

32. Arts RJW, Carvalho A, La Rocca C, Palma C, Rodrigues F, Silvestre R, et al. Immunometabolic pathways in BCG-induced trained immunity. *Cell Rep* (2016) 17 (10):2562–71. doi: 10.1016/j.celrep.2016.11.011

33. Dominguez-Andres J, Novakovic B, Li Y, Scicluna BP, Gresnigt MS, Arts RJW, et al. The itaconate pathway is a central regulatory node linking innate immune tolerance and trained immunity. *Cell Metab* (2019) 29(1):211–20 e5. doi: 10.1016/j.cmet.2018.09.003

34. Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Criado FJ, Miron-Canelo JA, Gil-Vicente A, Nieto-Huertos A, et al. Evaluation of a therapeutic vaccine for the prevention of recurrent urinary tract infections versus prophylactic treatment with antibiotics. *Int Urogynecol J* (2013) 24(1):127–34. doi: 10.1007/s00192-012-1853-5

35. Yang B, Foley S. First experience in the UK of treating women with recurrent urinary tract infections with the bacterial vaccine Uromune((R)). *BJU Int* (2018) 121(2):289–92. doi: 10.1111/bju.14067

36. Lorenzo-Gomez MF, Padilla-Fernandez B, Garcia-Cenador MB, Virseda-Rodriguez AJ, Martin-Garcia I, Sanchez-Escudero A, et al. Comparison of sublingual therapeutic vaccine with antibiotics for the prophylaxis of recurrent urinary tract infections. *Front Cell Infect Microbiol* (2015) 5:50. doi: 10.3389/fcimb.2015.00050

37. Ramirez Sevilla C, Gomez Lanza E, Manzanera JL, Martin JAR, Sanz MAB. Active immunoprophyilaxis with uromune(R) decreases the recurrence of urinary tract infections at three and six months after treatment without relevant secondary effects. *BMC Infect Dis* (2019) 19(1):901. doi: 10.1186/s12879-019-4541-y

38. Carrion-Lopez P, Martinez-Ruiz J, Libran-Garcia L, Gimenez-Bachs JM, Pastor-Navarro H, Salinas-Sanchez AS. Analysis of the efficacy of a sublingual bacterial vaccine in the prophylaxis of recurrent urinary tract infection. *Urol Int* (2020) 104(3-4):293–300. doi: 10.1159/000505162

39. Lorenzo-Gomez MF, Padilla-Fernandez B, Flores-Fraile J, Valverde-Martinez S, Gonzalez-Casado I, Hernandez JD, et al. Impact of whole-cell bacterial immunoprophylaxis in the management of recurrent urinary tract infections in the frail elderly. *Vaccine* (2021) 39(42):6308–14. doi: 10.1016/j.vaccine.2021.08.093

40. Sanchez-Ramon S, Fernandez-Paredes L, Saz-Leal P, Diez-Rivero CM, Ochoa-Grullon J, Morado C, et al. Sublingual bacterial vaccination reduces recurrent infections in patients with autoimmune diseases under immunosuppressant treatment. *Front Immunol* (2021) 12:675735. doi: 10.3389/fimmu.2021.675735

41. Lorenzo-Gómez M-F, Foley S, Nickel JC, García-Cenador M-B, Padilla-Fernández B-Y, González-Casado I, et al. Sublingual MV140 for prevention of recurrent urinary tract infections. *NEJM Evid* (2022) 1(4):EVIDoa2100018. doi: 10.1056/EVIDoa2100018

42. Dominguez-Andres J, Arts RJW, Bekkering S, Bahrar H, Blok BA, de Bree LCJ, et al. *In vitro* induction of trained immunity in adherent human monocytes. *STAR Protoc* (2021) 2(1):100365. doi: 10.1016/j.xpro.2021.100365

43. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection *via* functional reprogramming of monocytes. *Cell Host Microbe* (2012) 12(2):223–32. doi: 10.1016/j.chom.2012.06.006

44. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille calmette-guerin induces NOD2-dependent nonspecific protection from reinfection *via* epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A* (2012) 109(43):17537–42. doi: 10.1073/pnas.1202870109

45. Novakovic B, Habibi E, Wang SY, Arts RJW, Davar R, Megchelenbrink W, et al. Beta-glucan reverses the epigenetic state of LPS-induced immunological tolerance. *Cell* (2016) 167(5):1354–68 e14. doi: 10.1016/j.cell.2016.09.034

46. Saeed S, Quintin J, Kerstens HH, Rao NA, Aghajanirefah A, Matarese F, et al. Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science* (2014) 345(6204):1251086. doi: 10.1126/science.1251086

47. Foxman B, Muraglia R, Dietz JP, Sobel JD, Wagner J. Prevalence of recurrent vulvovaginal candidiasis in 5 European countries and the united states: results from an internet panel survey. *J Low Genit Tract Dis* (2013) 17(3):340–5. doi: 10.1097/LGT.0b013e318273e8cf

48. Brodie A, El-Taji O, Jour I, Foley C, Hanbury D. A retrospective study of immunotherapy treatment with uro-vaxom (OM-89(R)) for prophylaxis of recurrent urinary tract infections. *Curr Urol* (2020) 14(3):130–4. doi: 10.1159/000499248

49. Smith AL, Brown J, Wyman JF, Berry A, Newman DK, Stapleton AE. Treatment and prevention of recurrent lower urinary tract infections in women: A rapid review with practice recommendations. *J Urol* (2018) 200(6):1174–91. doi: 10.1016/j.juro.2018.04.088

50. Edwards JEJr., Schwartz MM, Schmidt CS, Sobel JD, Nyirjesy P, Schodel F, et al. A fungal immunotherapeutic vaccine (NDV-3A) for treatment of recurrent vulvovaginal candidiasis-a phase 2 randomized, double-blind, placebo-controlled trial. *Clin Infect Dis* (2018) 66(12):1928–36. doi: 10.1093/cid/ciy185

51. Farr A, Effendy I, Frey Tirri B, Hof H, Mayser P, Petricevic L, et al. Guideline: Vulvovaginal candidosis (AWMF 015/072, level S2k). *Mycoses* (2021) 64 (6):583–602. doi: 10.1111/myc.13248

52. Tomas M, Rolo J, Gaspar C, Palmeira-de-Oliveira A, Simoes S, Katz DF, et al. Sodium bicarbonate gels: a new promising strategy for the treatment of vulvovaginal candidosis. *Eur J Pharm Sci* (2021) 157:105621. doi: 10.1016/j.ejps.2020.105621

53. Benn CS, Fisker AB, Rieckmann A, Sorup S, Aaby P. Vaccinology: time to change the paradigm? *Lancet Infect Dis* (2020) 20(10):e274–e83. doi: 10.1016/S1473-3099(19)30742-X

54. Angelidou A, Diray-Arce J, Conti MG, Netea MG, Blok BA, Liu M, et al. Human newborn monocytes demonstrate distinct BCG-induced primary and trained innate cytokine production and metabolic activation. *In Vitro Front Immunol* (2021) 12:674334. doi: 10.3389/fimmu.2021.674334

55. Smeekens SP, Gresnigt MS, Becker KL, Cheng SC, Netea SA, Jacobs L, et al. An anti-inflammatory property of candida albicans beta-glucan: Induction of high levels of interleukin-1 receptor antagonist *via* a dectin-1/CR3 independent mechanism. *Cytokine* (2015) 71(2):215–22. doi: 10.1016/j.cyto.2014.10.013

56. Gresnigt MS, Bozza S, Becker KL, Joosten LA, Abdollahi-Roodsaz S, van der Berg WB, et al. A polysaccharide virulence factor from aspergillus fumigatus elicits anti-inflammatory effects through induction of interleukin-1 receptor antagonist. *PloS Pathog* (2014) 10(3):e1003936. doi: 10.1371/journal.ppat.1003936

57. Arts RJW, Moorlag S, Novakovic B, Li Y, Wang SY, Oosting M, et al. BCG Vaccination protects against experimental viral infection in humans through the induction of cytokines associated with trained immunity. *Cell Host Microbe* (2018) 23(1):89–100 e5. doi: 10.1016/j.chom.2017.12.010

58. Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, et al. Trained immunity or tolerance: opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. *Clin Vaccine Immunol* (2014) 21(4):534–45. doi: 10.1128/CVI.00688-13

59. Del Fresno C, Garcia-Arriaza J, Martinez-Cano S, Heras-Murillo I, Jarit-Cabanillas A, Amores-Iniesta J, et al. The bacterial mucosal immunotherapy MV130 protects against SARS-CoV-2 infection and improves COVID-19 vaccines immunogenicity. *Front Immunol* (2021) 12:748103. doi: 10.3389/fimmu.2021.748103

60. Arts RJ, Blok BA, Aaby P, Joosten LA, de Jong D, van der Meer JW, et al. Long-term *in vitro* and *in vivo* effects of gamma-irradiated BCG on innate and adaptive immunity. *J Leukoc Biol* (2015) 98(6):995–1001. doi: 10.1189/jlb.4MA0215-059R

61. Arts RJ, Blok BA, van Crevel R, Joosten LA, Aaby P, Benn CS, et al. Vitamin a induces inhibitory histone methylation modifications and down-regulates trained immunity in human monocytes. *J Leukoc Biol* (2015) 98(1):129–36. doi: 10.1189/jlb.6AB0914-416R

62. Brandi P, Conejero L, Cueto FJ, Martinez-Cano S, Dunphy G, Gomez MJ, et al. Trained immunity induction by the inactivated mucosal vaccine MV130 protects against experimental viral respiratory infections. *Cell Rep* (2022) 38 (1):110184. doi: 10.1016/j.celrep.2021.110184

63. Vierboom MPM, Dijkman K, Sombroek CC, Hofman SO, Boot C, Vervenne RAW, et al. Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination. *Cell Rep Med* (2021) 2 (1):100185. doi: 10.1016/j.xcrm.2020.100185

64. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med* (2005) 11(4 Suppl):S45–53. doi: 10.1038/nm1213

65. Negri DR, Riccomi A, Pinto D, Vendetti S, Rossi A, Cicconi R, et al. Persistence of mucosal and systemic immune responses following sublingual immunization. *Vaccine* (2010) 28(25):4175–80. doi: 10.1016/j.vaccine.2010.04.013

66. Lavelle EC, Ward RW. Mucosal vaccines - fortifying the frontiers. *Nat Rev Immunol* (2022) 22(4):236–50. doi: 10.1038/s41577-021-00583-2

Check for updates

Received: 16 August 2022 | Revised: 4 November 2022 | Accepted: 20 November 2022

DOI: 10.1111/cea.14261

REVIEW ARTICLE

WILEY

From trained immunity in allergy to trained immunity-based allergen vaccines

Leticia Martín-Cruz[1] | Carmen Sevilla-Ortega[1] | Alba Angelina[1] | Jorge Domínguez-Andrés[2,3] | Mihai G. Netea[2,3,4] | José Luis Subiza[5] | Oscar Palomares[1]

[1]Department of Biochemistry and Molecular Biology, School of Chemistry, Complutense University of Madrid, Madrid, Spain

[2]Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Centre, Nijmegen, The Netherlands

[3]Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands

[4]Department of Immunology and Metabolism, Life & Medical Sciences Institute, University of Bonn, Bonn, Germany

[5]Inmunotek, Alcalá de Henares, Madrid, Spain

Correspondence
Oscar Palomares, Department of Biochemistry and Molecular Biology, School of Chemistry, Complutense University of Madrid, Avenida Complutense s/n, 28040 Madrid, Spain.
Email: oscar.palomares@quim.ucm.es

Funding information
Ministerio de Educación y Cultura

Abstract

Innate immune cells experience long lasting metabolic and epigenetic changes after an encounter with specific stimuli. This facilitates enhanced immune responses upon secondary exposition to both the same and unrelated pathogens, a process termed trained immunity. Trained immunity-based vaccines (TIbV) are vaccines able to induce innate immune memory, thus conferring heterologous protection against a broad range of pathogens. While trained immunity has been well documented in the context of infections and multiple immune-mediated diseases, the role of innate immune memory and its contribution to the initiation and maintenance of chronic allergic diseases remains poorly understood. Over the last years, different studies attempting to uncover the role of trained immunity in allergy have emerged. Exposition to environmental factors impacting allergy development such as allergens or viruses induces the reprogramming of innate immune cells to acquire a more pro-inflammatory phenotype in the context of asthma or food allergy. Several studies have convincingly demonstrated that prevention of viral infections using TIbV contributes to reduce wheezing attacks in children, which represent a high-risk factor for asthma development later in life. Innate immune cells trained with specific stimuli might also acquire anti-inflammatory features and promote tolerance, which may have important implications for chronic inflammatory diseases such as allergies. Recent findings showed that allergoid-mannan conjugates, which are next generation vaccines for allergen-specific immunotherapy (AIT), are able to reprogram monocytes into tolerogenic dendritic cells by mechanisms depending on metabolic and epigenetic rewiring. A better understanding of the underlying mechanisms of trained immunity in allergy will pave the way for the design of novel trained immunity-based allergen vaccines as potential alternative strategies for the prevention and treatment of allergic diseases.

KEYWORDS
allergy, asthma, basic immunology, clinical immunology, food allergy, innate immunity, trained immunity, trained immunity-based allergen vaccines

Leticia Martín-Cruz and Carmen Sevilla-Ortega equally contributed to this work.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2022 The Authors. *Clinical & Experimental Allergy* published by John Wiley & Sons Ltd.

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

1 | INTRODUCTION

Although the immune memory has been classically associated with the adaptive immune system, in the recent years several studies convincingly demonstrated that innate immune cells also exhibit memory. Trained immunity was defined by Netea and colleagues in 2011 as the process by which innate immune cells display long-term functional reprogramming after an encounter with a primary stimulus, resulting in an enhanced immune response against a secondary challenge.[1] After recognition of the first stimulus, innate immune cells (monocytes, macrophages, NK cells, neutrophils, dendritic cells (DCs) and innate lymphoid cells (ILCs)) and bone marrow progenitors undergo metabolic and epigenetic reprogramming, two major molecular mechanisms responsible for the induction of trained immunity.[2] This process has also important applications in modern vaccinology. Trained immunity-based vaccines (TIbV) are defined as vaccines able to induce trained immunity, thus stimulating broader responses against both related and unrelated antigens contained in the vaccine formulation.[3,4]

Together with infections, allergic diseases are a major health problem whose prevalence is increasing around the world, mainly due to environmental factors, behavioural modifications or changes in lifestyle.[5,6] Induction of trained immunity could play a central role within the mechanisms involved in the initiation and chronification of allergy, but data related to the actual role of trained innate cells in allergy are scarce. Several studies reported that some allergens, virus, and other stimuli mediate training mechanisms that might contribute to the deleterious effects and aberrant innate immune responses reported in asthma and food allergy.[7–9] In contrast, some bacterial preparations promote trained immunity granting protection against respiratory viral infections and wheezing attacks in children, which represent a high-risk factor for later asthma development.[10,11] It has been suggested that allergen-specific immunotherapy (AIT) might induce trained immunity as a relevant mechanism contributing to the restoration of healthy immune responses to allergens.[12] In this regard, allergoid-mannan conjugates are able to reprogram monocytes into tolerogenic DCs through mechanisms depending on metabolic and epigenetic reprogramming. This suggests that this next generation AIT vaccine might have the potential to induce trained immunity.[13] However, the detailed molecular mechanisms by which trained immunity contributes to allergy development and to successful AIT remain largely unknown. In this article, we comprehensively review the most recent findings related to the role of trained immunity in the context of allergy, which might pave the way for the development of future novel TIbV for AIT as potential novel therapeutic strategies for allergic diseases.

Key messages

- Trained immunity-based vaccines induces innate immune memory providing heterologous protection against different pathogens.
- Environmental factors driving allergy promote training of innate cells enhancing type 2 inflammatory responses.
- Allergoid-mannan conjugates AIT vaccines reprogram monocytes into tolerogenic DCs by promoting metabolic and epigenetic rewiring.

2 | TRAINED IMMUNITY: DEFINITION, CELLS AND UNDERLYING MECHANISMS

The field of innate immune memory, also termed 'trained immunity', started one decade ago, when two studies respectively described the existence of enhanced responsiveness after sublethal *Candida* infection in mice, and the presence of increased cytokine production after the stimulation of peripheral blood mononuclear cells obtained from individuals vaccinated with the *Bacille Calmette-Guérin* (BCG).[14,15] In the last years, we have witnessed a quick expansion of this field, with multiple studies describing a variety of mechanisms, stimuli inducing trained immunity, development of experimental models, and potential applications, besides showing the existence of these processes in multiple cell models and organisms (for review, see Ref. [2]). In this rapidly expanding context, it is important to remind the definition of trained immunity and distinguish it from other related immunological processes such as priming or tolerance.[16] During trained immunity, an initial stimulus induces long-term epigenetic and metabolic changes that render the cells more responsive to secondary stimulation (Figure 1). Initially, trained immunity was described in differentiated innate immune cells such as monocytes, macrophages, and NK cells.[2] However, other cell types such as epithelial cells can also be trained.[17] Among the different cell subsets that can undergo this process, there is one that stands out: the haematopoietic stem cells of the bone marrow, which transmit their trained phenotype to their daughter cells allowing the long-term establishment of trained immunity responses that can last for weeks, months, and possible even years.[18,19] These mechanisms allow that those immune responses that initially occur locally are transmitted to the rest of the body and generate long-term systemic immunity.

Importantly, after the initial stimulation ceases, the immune response goes back to the steady state. Though the cells are trained, there is no maintained secretion of cytokines or chemokines (Figure 1). Nevertheless, trained cells undergo strong epigenetic and metabolic reprogramming, allowing them to present enhanced responsiveness upon secondary stimulation.[20,21] In contrast to adaptive immune responses, the secondary stimulus that triggers innate immune memory can be of the same or a different nature than the first one, so trained immunity induces heterologous, antigen-independent secondary enhanced responses (Figure 1).

As mentioned before, the induction and maintenance of trained immunity relies on two main pillars: the epigenetic and metabolic reprogramming of the cells. Trained cells increase their basal metabolic activities so they can use nutrients and energy faster and more efficiently.[22] In this regard, they quickly rewire their glycolytic and

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

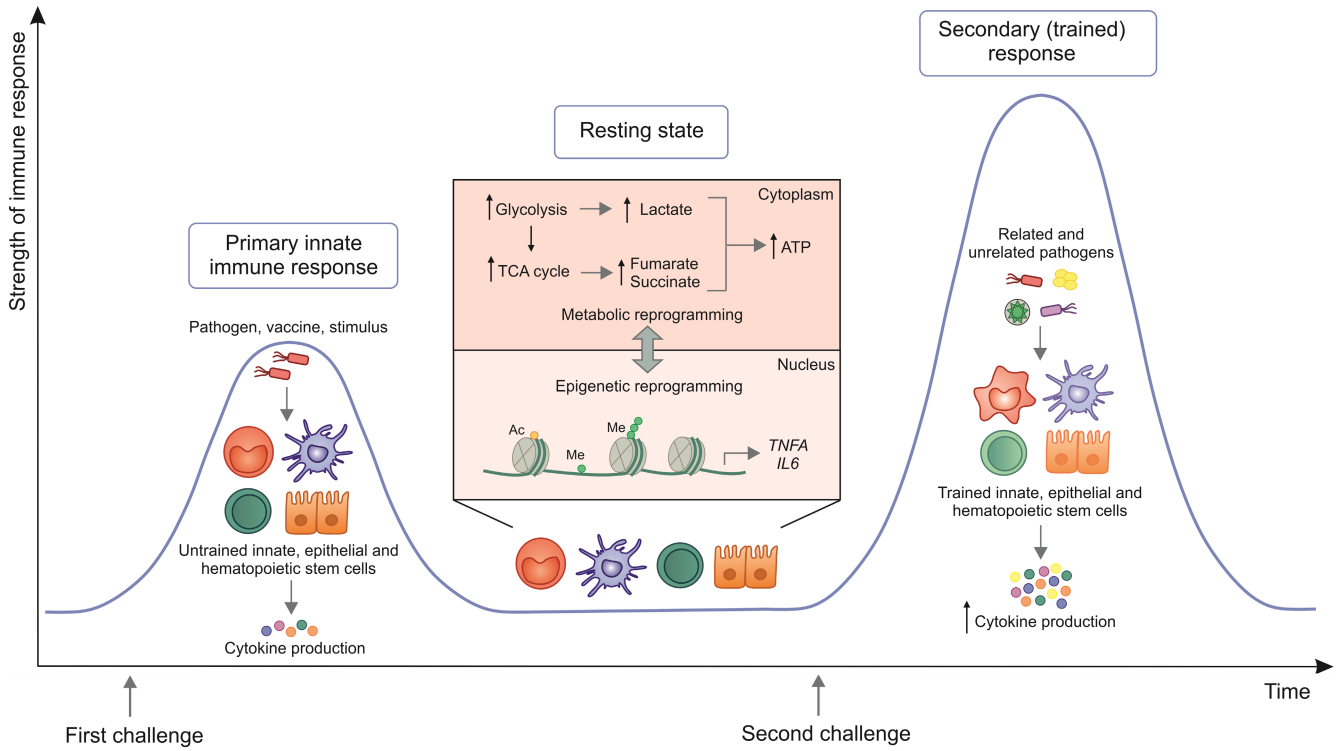

FIGURE 1 Induction of trained immunity. Different pathogens, vaccines and stimuli induce long-term functional, metabolic and epigenetic reprogramming of innate, epithelial and haematopoietic stem cells. Trained immunity facilitates enhanced responsiveness after a second challenge with related or unrelated pathogens conferring heterologous protection. TCA cycle, tricarboxylic acid cycle; ATP, adenosine triphosphate; Ac, acetylation; Me, methylation

oxidative phosphorylation activities, by increasing their glucose consumption, leading to enhanced production of lactate and ATP.[23–25] This is paralleled by an increased amino acid and fatty acid consumption, so the cells extract the most of their available nutrients, using the necessary building blocks and energy to fuel and maintain the intracellular processes required for enhanced immune responsiveness.[24] This increased metabolic activity is accompanied by the remodelling of the epigenetic architecture of the cell (Figure 1). The modification of the acetylation and methylation marks in the histones and the DNA remodels the three-dimensional structure of the chromatin in the cell nucleus.[26] Through these mechanisms, the promoter regions of pro-inflammatory genes such as *TNFA* or *IL6* maintain an open conformation after the first stimulus ceases, so the transcriptional machinery of the cell has a facilitated access to these genome regions. Similar processes have been suggested to mediate long-term enhanced type 2 responses important in allergies in ILC2 cells.[27]

The combination of an increased metabolic activity and the enhanced accessibility to several pro-inflammatory regions of the genome promotes the quick and enhanced transcription, translation, processing and secretion of the necessary factors to mount an immune response. Moreover, rewiring the cell metabolism induces the production and accumulation of multiple metabolites with direct immunomodulatory activities, such as succinate, fumarate, or lactate.[22] Accumulation of these metabolites contributes to amplification or modulation of trained immunity through inhibiting or activating multiple enzymes and pathways fundamental for immune responses and epigenetic processes. Therefore, the interplay between the metabolic and epigenetic rewiring of the cells is fundamental for the induction, maintenance, and regulation of trained immunity (Figure 1).

Innate immune tolerance, as opposite to trained immunity, is defined as the process by which innate cells are unable to activate gene transcription, preventing them from carrying out their functions after restimulation, such as macrophages upon repeated or sustained stimulation with LPS.[16] In contrast, specific microbial products such as those derived from certain helminths might induce a so-called 'anti-inflammatory' trained immunity phenotype characterized by epigenetic and metabolic reprograming leading to the production of higher levels of anti-inflammatory cytokines such as IL-10 or IL-1RA. This might have important implications in the context of allergy as discussed in more detail in the following sections.[28]

3 | TRAINED IMMUNITY-BASED VACCINES (TIBV) AS A NEW PARADIGM IN VACCINOLOGY

The change in paradigm towards the recognition of innate immune memory as an important mechanism contributing to immunological memory has opened a new avenue in modern vaccinology.[29,30] While traditional vaccines promote adaptive immune responses acting on

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

antigen-specific memory B and T cells,[31] trained immunity-based vaccines (TIbV) are defined as vaccines able to induce trained immunity, thus stimulating broader responses against both related and unrelated antigens contained in the vaccine formulations.[3] Different vaccine preparations containing lived attenuated or inactivated pathogens might confer protection against target infections, but also exhibit heterologous effects by mechanisms combining enhanced innate and adaptive immune responses (Figure 2).[3,32]

The paradigmatic example of TIbV is the live attenuated *Mycobacterium bovis* BCG vaccine, which has been shown to enhance both antigen-specific T cell responses and non-specific innate immune responses.[33,34] BCG administration in healthy adults induced heterologous responses in monocytes and NK cells in response to mycobacteria and other unrelated pathogens such as *Staphylococcus aureus* or *Candida albicans* even 3 months after vaccination.[15,35] BCG vaccination also imprints trained immunity properties in bone marrow haematopoietic stem cells and multipotent progenitors, which are essential to maintain the memory of circulating innate immune cells in blood and tissues.[18] Epidemiological studies have convincingly demonstrated that BCG vaccination reduces all-cause mortality in children[36,37] and increases the time to the first infection in the elderly.[38] TIbV also provide protection against viral infections and, therefore, BCG has been suggested as a potential alternative for SARS-CoV-2 virus infection with several clinical trials already published and others still ongoing.[39,40] Other live attenuated formulations including measles-containing vaccines, oral polio, vaccinia virus and influenza vaccines provide protection against both homologous and heterologous diseases by promoting trained immunity. Therefore, could be also considered as conventional vaccines displaying features related to TIbV.[41–45]

Interestingly, different types of bacterial formulations for recurrent respiratory tract infections (RRTIs) confer protection against a broad range of infectious diseases. Clinical data showed that OM-85, a formulation of 21 bacterial strain lysates from five genera (*Moraxella*, *Haemophilus*, *Klebsiella*, *Staphylococcus* and *Streptococcus*), reduced the infection rates in children and adults with RRTIs.[46,47] In the same line, clinical studies demonstrated that MV130, a whole-heat inactivated polybacterial preparation containing 90% of Gram-positive (*Streptococcus pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*) and a 10% of Gram-negative (*Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae*) bacteria, decreased the incidence and severity of RRTIs in adults.[48,49] A randomized, double-blind, placebo-controlled phase III clinical trial also demonstrated that sublingual MV130 prevents recurrent wheezing in children even after 6 months of treatment discontinuation.[50] MV140, a whole heat-inactivated formulation with 75% Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae and Proteus vulgaris*) and 25% Gram-positive (*Enterococcus faecalis*) bacteria, prevented recurrent urinary tract infections and promoted Th1/Th17 and IL-10 immune responses.[51–53] The combination of MV140 with heat-inactivated *Candida albicans* (V132) in a single preparation induced metabolic and epigenetic reprograming in human DCs and enhanced antigen specific T cell responses.[54]

Several studies have shown that TIbV may be also employed to treat cancer and some autoimmune diseases such as multiple sclerosis or type 1 diabetes.[55,56] In addition, BCG is a standard treatment for bladder cancer and the positive outcomes have been ascribed to the induction of trained immunity.[55] BCG vaccination has important clinical benefits for multiple sclerosis with decreased tissue damage reported even 5 years after BCG vaccination.[57,58] Mechanistically, BCG enhances glycolysis in conventional T cells, which in turn favours the generation of suppressive regulatory T cells (Tregs) that prevent neuronal damage.[55,59] BCG-mediated enhanced glycolysis also contributes to the control of blood sugar levels in type 1 diabetes.[56,60]

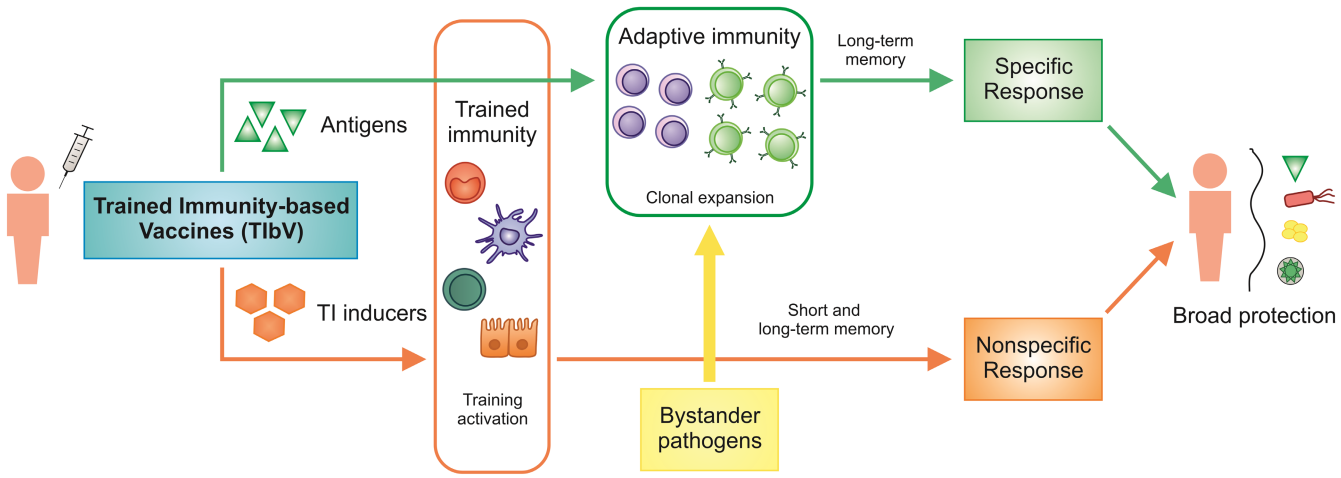

FIGURE 2 Mechanisms of trained immunity-based vaccines (TIbV) for infections. TIbV are formed by two essential components: Trained Immunity (TI) inducers and TIbV-related antigens. TI inducers reprogram innate, epithelial and haematopoietic stem cells so that they can respond in a much more efficient manner against bystander pathogens (both related and unrelated to those contained in the formulation), thus resulting in a short/long-lasting memory. Under TI conditions, more potent adaptive responses are orchestrated by T and B cells against both the antigens contained in the vaccine formulation but also against antigens contained in encountered bystander pathogens. TIbV are able to confer broad protection against both related and unrelated bystander pathogens.

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

While the concept of trained immunity is generally linked to higher responses after a secondary stimulation, trained innate immune cells might also acquire and promote anti-inflammatory and tolerogenic profiles depending on the type of stimuli, the concentration and duration of the stimulation, the type of TIbV or the specific pathological context.[16,56,61] The understanding of the potential role of trained immunity in the initiation and maintenance of inflammatory immune-mediated diseases such as asthma and allergy is improving over the last years, which might well pave the way for the development of novel interventions to prevent and/or restore proper immune responses.

4 | TRAINED IMMUNITY IN ALLERGY

Allergic diseases represent a global health problem of increasing prevalence with huge socio-economic impact worldwide.[62,63] Allergic asthma, allergic rhinitis, food allergy, atopic eczema/dermatitis and anaphylaxis represent the most important chronic allergic diseases with a significant negative impact on patients' quality of life and health-associated costs. The immunological mechanisms underlying allergy can be divided into two phases: sensitization/memory and effector phase. Sensitization occurs during the first contact with the allergen and leads to the generation of allergen-specific $CD4^+$ Th2 cells and the production of allergen-specific IgE antibodies by B cells. Allergen-specific IgE binds to the high-affinity FcЄRI receptor on mast cells and basophils, thus leading to patient's sensitization.[64–66] New encounters with the allergen cause cross-linking of IgE-FcεRI complexes on sensitized mast cells and basophils, releasing a plethora of anaphylactic mediators that trigger the immediate clinical symptoms.[64–66] Accumulation of anaphylactic mediators and activation of allergen-specific memory Th2 cells via IgE-facilitated presentation by DC and B cells initiates late-phase reactions. IL-4, IL-5, IL-9 and IL-13 produced by Th2 cells in cooperation with ILC2s activated by epithelial alarmins (TSLP, IL-33 or IL-25) contribute to allergen-specific IgE levels, eosinophilia, mucus production, inflammatory cell recruitment and tissue inflammation. These effects are associated with the chronicity and more severe clinical manifestations.[64] Westernized lifestyle and exposome including all environmental exposures such as chemicals, pollutants as well as lifestyle factors, dietary habits, drugs and infections agents are important risk factors for allergic sensitization.[6,67,68] This association suggests the important role of gene-environmental interactions, supported by epigenetic and metabolic rewiring, in the development of allergic diseases.[69,70] Therefore, a better understanding of the potential role of trained immunity in the context of allergic diseases is of outmost importance for the development of future alternative interventions.

4.1 | Trained immunity in allergic asthma

Asthma is one of the most prevalent inflammatory airway diseases affecting more than 330 million people in the world.[71] Asthma is a heterogeneous syndrome characterized by reversible airway obstruction, bronchial hyperresponsiveness and inflammation, which progressively accelerate lung function decline and airway remodelling.[71,72] Allergic asthma constitutes one of the most common phenotypes and it is characterized by increased levels of total and allergen-specific serum IgE and type 2 immune responses.[64] Type 2 asthma onset is associated with early life exposure to allergens, pollutants, irritants or viral infections. Many children suffer from recurrent wheezing episodes triggered by rhinovirus or respiratory syncytial virus that can diminish with age, but for some individuals can mark the beginning of asthma.[73] As discussed above, the protective role of different bacterial preparations for preventing viral infections and wheezing attacks in children have been demonstrated in several clinical studies,[46,50] and the mechanisms underlying their mode of action are starting to be elucidated.[3,74] The bacterial lysate OM-85 increases protection against viral respiratory infections in murine models by enhancing mucosal antibody production.[10] Maternal oral OM-85 administration conferred protection to the offspring against neonatal respiratory viral infection by accelerating and enhancing innate immune responses.[75] In humans, oral OM-85 also prevented RRTI by mechanisms depending on immune training, but whether trained immunity is induced remains unknown.[76] Recently, it has been demonstrated that airway administration of OM-85 impairs asthma features by targeting DCs and IL-33-activated ILC2s.[77] The whole heat-inactivated polybacterial vaccine MV130 induced a robust activation of human DCs from healthy donors and patients suffering from RRTI, thus promoting the generation of potent Th1, Th17, and IL-10-producing T cells against both related and unrelated antigens contained in the vaccine formulation.[78] In mice models, it has been also demonstrated that intranasal administration of MV130 confers protection against viral infections by mechanisms depending on the induction of trained immunity via metabolic and epigenetic reprogramming of myeloid immune cells and bone marrow progenitor cells,[11] thus supporting the capacity of MV130 to prevent wheezing attacks in children previously reported in phase III clinical trials.[50]

Contrary to harmful effects widely associated with viral respiratory infections, it has been reported that murid gamma herpes virus (MuHV-4) infection prior to HDM experimental asthma induction, blocks asthma development.[79] MuHV-4 infection induces the death of lung resident alveolar macrophages and the subsequent repopulation of the lung niche by regulatory bone-marrow-derived monocytes. Locally differentiated monocyte-derived macrophages block type 2 HDM-induced specific response mediated by DCs, resulting in lower pro-asthmatic type 2 cytokines in both the lungs and mediastinal lymph nodes.[79] However, the mechanism by which MuHV-4 infection reprograms bone marrow-derived monocytes remains unknown.

Recently, mouse studies showed that ILC2s, critical players in type 2 asthma development, might also acquire memory in an IL-33 dependent manner.[80–82] Different stimuli such as IL-33, *Aspergillus* protease, rhinovirus infection or papain allergen drives IL-33 production by the epithelial cells, and consequently, this IL-33 leads to

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

the activation and proliferation of lung ILC2s. Following activation, a resting phase takes place with a decrease in ILC2 numbers in the lungs and a subsequent reduction of cytokine production. However, the re-stimulation with a second unrelated stimulus significantly enhances cytokine production by IL-33-trained ILC2s, which could be associated with an enhancement of allergic asthmatic inflammation (Figure 3).[7,8,27]

4.2 | Trained Immunity in food allergy

The prevalence of food allergy is rising in westernized countries, affecting approximately 6% of children and 4% of adults.[83,84] Food allergy arises from a breakdown of oral tolerance to ingested antigens in people genetically and possibly also environmentally predisposed to atopic disease.[83] Therefore, the association of food allergy prevalence with lifestyles suggests that trained immunity mechanisms could be also involved in the development and persistence of food allergy.[6]

Regarding the potential role of innate immune memory in food allergy, some studies have reported excessive innate immune responses at birth in cord blood-derived mononuclear cells from allergic children compared to non-allergic donors.[85,86] These responses were characterized by large amounts of IL-6, IL-1β and TNF-α production following in vitro Toll-like receptor stimulation. This study has reported that the predisposition for allergen-specific type 2 responses is associated with the hyperinflammatory reaction in the perinatal period.[85] To further confirm the hypothesis, naive $CD4^+$ T cells from cord blood were stimulated with IL-1β and TNF-α (in combination with TGF-β), which resulted in an enhanced type 2 immune response.[86] Recent studies have confirmed the enhancement of innate immune responses in egg- and peanut-allergic 1-year-old infants compared to healthy donors.[9,87,88] Egg-allergic paediatric patients showed a higher frequency of circulating monocytes and DCs. Moreover, monocytes stimulated with LPS produced higher amount of IL-6, IL-1β, IL-8 and TNF-α compared with non-allergic patients. However, levels of IL-12p70 were higher in non-allergic donors than in allergic children (before and after LPS stimulation).[87] Egg-allergic infants also displayed a lower number of $CD4^+$ Tregs and increased monocye:$CD4^+$ T cell ratio.[9] On the other hand, other study with peanut allergic infants demonstrated an increase in TNF-α production after non-specific stimulation of total peripheral blood mononuclear cells from one-year peanut-allergic donors compared to healthy infants.[88] To confirm whether the enhanced innate immune response remains with ageing, the same parameters were evaluated in single peanut or multi-food allergic adolescences.[89] Both single peanut and multi-food allergic adolescence displayed an increase in circulating levels of DCs and monocytes. A higher pro-inflammatory cytokine production after LPS stimulation of monocytes than in healthy donors was observed.[89] Collectively, all these results suggest an increase in innate immune response displaying trained immunity features, which might favour DC and T cell interaction favouring type 2 immune responses early in life in food-allergic patients that might persist through adolescence (Figure 4).[9,85–89]

4.3 | Trained Immunity in allergen-specific immunotherapy (AIT)

AIT is currently the only causative treatment for allergic diseases with potential disease-modifying capacity.[90] AIT consists of the administration of high doses of the causative allergens to induce patient's desensitization and allergen tolerance after treatment cessation. Research on the mechanisms of action of AIT has been traditionally focused on the regulation of adaptive immune responses, however, recent findings suggest that innate immune responses might also be regulated during successful AIT.[91] It has been shown that AIT restores the frequencies of different innate immune cell populations (ILCs, monocytes and DCs) to those observed in non-allergic individuals.[12] Whether metabolic and epigenetic reprogramming driving trained immunity was also induced by AIT in these restored innate immune cell populations was not reported in this study. At this regard, subcutaneous and sublingual AIT as well as grass pollen-induced seasonal allergic rhinitis changed the chromatin landscape in circulating follicular helper T cells (T_{FH}) and follicular Tregs (T_{FR}) compared with non-atopic patients and between groups.[92] T_{FH} from pollen allergic patients displayed more accessible chromatin regions associated with cell growth, differentiation,

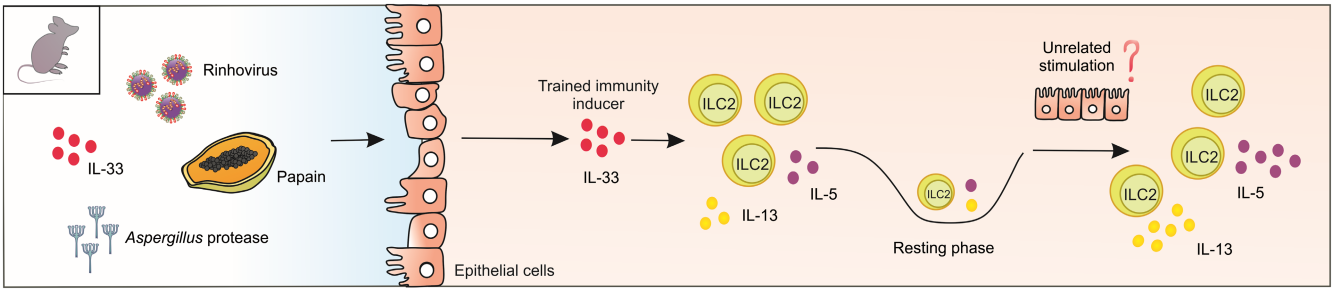

FIGURE 3 Induction of trained immunity in ILC2 under allergy conditions. Different stimuli (IL-33, papain, *Aspergillus* protease and rhinovirus) induce IL-33 production by epithelial cells, which stimulate type 2 innate lymphoid cells (ILC2) activation and cytokine production. After stimulation a resting phase takes place with less ILC2 that also produce less IL-13 and IL-5. Second unrelated stimulation of ILC2 results in an enhanced response that could be associated with a worst asthmatic reaction

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

and proliferation than T_{FR}. Remarkably, AIT modified this chromatin landscape and generated a more regulatory profile characterized by the induction of T_{FR} and IL10$^+$ T_{FH}.[92] Moreover, another clinical trial shows that sublingual AIT induces IL-10-producting ILCs in patients with grass-pollen allergy.[93] It has been also demonstrated that ILC2 can be converted into IL-10-producing ILC2, which plays a critical role in the restoration of epithelial cell integrity, suppression of type 2 responses and promotion of tolerance to aeroallergens.[94] This plasticity might be linked to epigenetic reviewing, but further research is needed to fully confirm it. The concept of trained immunity has been enlarged with reports showing that myeloid cells can be also trained in vitro or in vivo to be more anti-inflammatory following helminth exposure, which might confer protection against T cell-mediated autoimmunity.[28] Macrophages in vitro trained with *Fasciola hepatica* total extracts or *F. hepatica* excretory products (FHES) showed an increase in the anti-inflammatory cytokines IL-1RA and IL-10 and lower production of pro-inflammatory cytokines TNF-α and IL-12p70. In vivo administration of FHES resulted in hyporesponsive monocytes due to alterations of the haematopoietic stem cell niche in the bone marrow.[28] The administration of helminth products produced an 'anti-inflammatory' trained immunity associated with reduced Th1 and Th17 responses.[28] Whether such helminth products could be also exploited in the field of AIT to restore healthy immune responses to allergens remains unknown. Up to date, the data related to allergen vaccines able to induce 'anti-inflammatory' trained immunity are still scarce. In this regard, allergoid-mannan conjugates, next generation AIT vaccines targeting DCs and promoting the generation of functional Tregs,[95] are able to reprogram monocytes from allergic and healthy donors into tolerogenic DCs with higher capacity to prime functional FOXP3$^+$ Tregs.[13] Allergoid-mannan conjugates imprint epigenetic and metabolic rewiring in monocyte-derived DCs that ensures an open chromatin status in tolerogenic genes and shifts metabolism into mitochondrial oxidative phosphorylation, thus suggesting that training immunity mechanisms might also take place and contribute to the beneficial reported effects of this next generation AIT vaccine.[13]

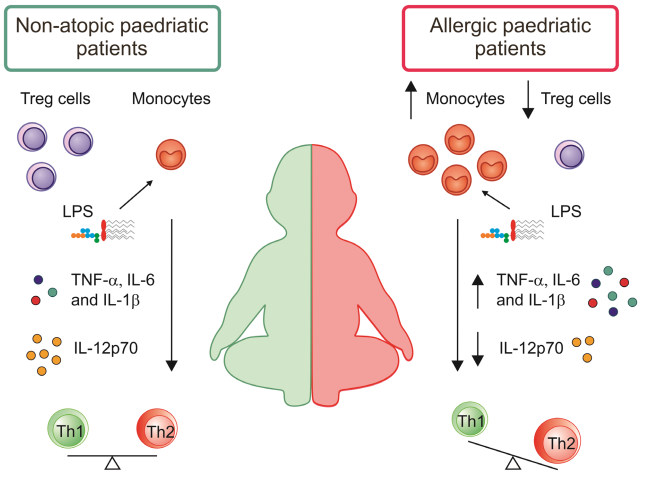

FIGURE 4 Food allergic paediatric patients display early hyperinflammatory innate immune responses that are associated with type 2 immunity. Allergic patients show less baseline Treg frequency and IL-12p70 production than healthy children. Children with food allergy also display larger number of monocytes producing higher amounts of IL-6, IL-1β and TNF-α after LPS stimulation than monocytes from non-allergic children. Those differences between groups skew the balance in favour to type 2 responses in allergic patients while maintaining a Th1:Th2 balance in healthy children. Treg cells, regulatory T cells; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor alpha

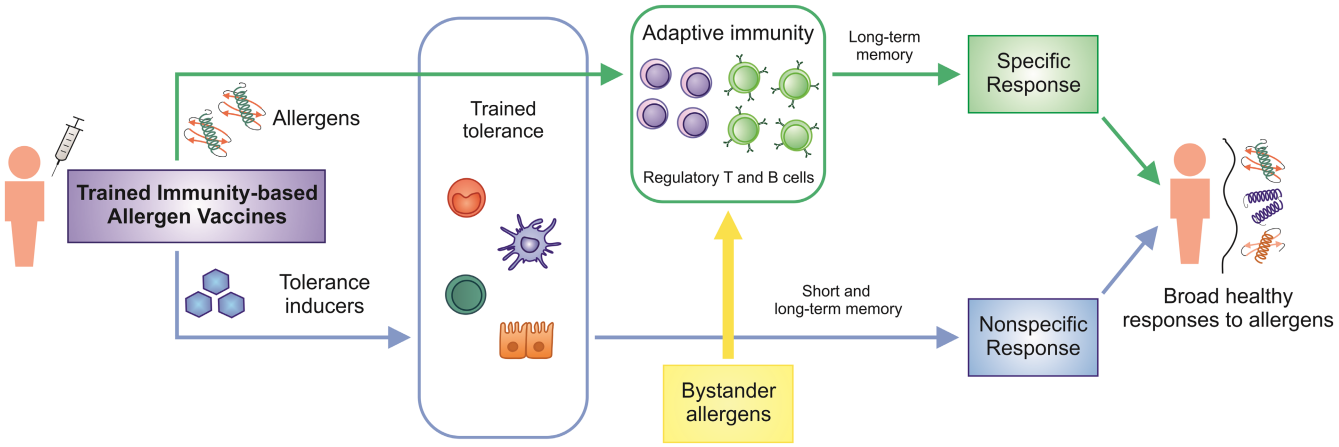

FIGURE 5 Proposed mode of action of trained immunity-based allergen vaccines. Trained immunity-based allergen vaccines should contain two essential components: Tolerance inducers and specific allergens to which the patients are sensitized to. These components will reprogram innate, epithelial, and haematopoietic stem cells so that they can mount anti-inflammatory and tolerogenic responses against bystander allergens (both related and unrelated to those contained the formulation), thus resulting in a short/long-lasting memory. Under these conditions, more potent tolerogenic adaptive responses are orchestrated by T and B cells against both the allergens contained in the vaccine formulation but also against encountered bystander allergens. Trained immunity-based allergen vaccines can induce broad healthy immune responses to allergens in a non-specific and allergen-specific manner, thus conferring broad tolerance against related and bystander allergens

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

5 | CONCLUSION AND FUTURE PERSPECTIVES

Our knowledge of the molecular mechanisms underlying trained immunity has significantly improved over the last decade. Nowadays it is widely accepted that innate immune cells also contribute to immunological memory. This change in paradigm has opened new alternatives in modern vaccinology. The use of TIbV has emerged as a useful approach to treating recurrent infections caused by related and heterologous pathogens. Different studies convincingly demonstrate that TIbV can be also successfully employed in other immune-mediated diseases such as cancer or autoimmunity. Recent findings showed that innate immune cells trained with a specific stimulus, such as helminth- and other specific bacterial- or yeast-derived products, might also acquire anti-inflammatory features and promote tolerance, which may have important implications for chronic inflammatory diseases such as allergies. Although the potential contribution of trained immunity to the development and chronification of allergic diseases such as asthma or food allergy is starting to be elucidated, the actual role of trained innate immune cells, epithelial cells or haematopoietic stem cell precursors in these processes still remains largely unknown. From a preventive and therapeutic point of view, TIbV formulated with certain polybacterial components showed efficacy in preventing RRTI, including viral-induced wheezing attacks in children, which represent a risk factor for asthma development. The restoration of proper innate immune responses might be also of great relevance during successful AIT. To date, allergen vaccines have not convincingly demonstrated yet capacity to induce trained immunity during AIT. In this regard, it has been previously shown that next generation allergoid-mannan conjugates reprogram monocytes into tolerogenic DCs by mechanisms depending on metabolic and epigenetic reprogramming, suggesting that this next generation AIT vaccine might well promote additional trained tolerance mechanisms associated with the reported clinical effects. However, further research is still needed to firmly demonstrate this capacity for allergoid-mannan conjugates. The better understanding of the potential role of trained immunity in the initiation and maintenance of allergy is of utmost importance as it will help to pave the way for the development of future novel TIbV for AIT (Figure 5), which might well contribute to improving preventive and/or therapeutic strategies for allergic diseases.

AUTHOR CONTRIBUTIONS

O.P. conceptualized and designed the scope of the review. L.M.-C., C.S.-O., A.A., J.D.-A., M.G.N., J.L.S. and O.P. wrote the manuscript. All the authors approved the final version of the paper.

ACKNOWLEDGEMENTS

The authors' laboratory is supported by grant PID2020-114396RB-I00 to O.P. from MICINN, Spain, The Netherlands Organization for Scientific Research (VENI grant 09150161910024 and Off Road grant 04510012010022) to JDA; ERC Advanced Grant (#833247) and a Spinoza Grant of the Netherlands Organization for Scientific Research to MGN. L.M.-C. was recipient of a FPU fellowship from MINECO, C.S.-O. is recipient of a predoctoral fellowship from "Doctorado Industrial between UCM-Inmunotek # IND2019/BMD-17182 by CAM. A.A. is a postdoctoral researcher ascribed to the project RTC2019-007097-1 by MICINNIN.

CONFLICT OF INTEREST

O.P. has received fee for lectures or participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, Diater, GSK, Pfizer, Inmunotek SL, Novartis, Sanofi Genzyme, Stallergenes and Regeneron. O.P. has received research grants from Inmunotek SL, Novartis SL, MINECO, MICINNIN and CAM. JLS is the founder and CEO of Inmunotek SL. The rest of the authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Leticia Martín-Cruz https://orcid.org/0000-0002-2546-1183
Carmen Sevilla-Ortega https://orcid.org/0000-0003-3072-9903

REFERENCES

1. Netea MG, Quintin J, van der Meer JW. Trained immunity: a memory for innate host defense. *Cell Host Microbe*. 2011;9(5):355-361.
2. Netea MG, Dominguez-Andres J, Barreiro LB, et al. Defining trained immunity and its role in health and disease. *Nat Rev Immunol*. 2020;20(6):375-388.
3. Sanchez-Ramon S, Conejero L, Netea MG, Sancho D, Palomares O, Subiza JL. Trained immunity-based vaccines: a new paradigm for the development of broad-Spectrum anti-infectious formulations. *Front Immunol*. 2018;9:2936.
4. Netea MG, van Crevel R. BCG-induced protection: effects on innate immune memory. *Semin Immunol*. 2014;26(6):512-517.
5. Simon D. Recent advances in clinical allergy and immunology. *Int Arch Allergy Immunol*. 2018;177(4):324-333.
6. Akdis CA. Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? *Nat Rev Immunol*. 2021;21(11):739-751.
7. Martinez-Gonzalez I, Matha L, Steer CA, Ghaedi M, Poon GF, Takei F. Allergen-experienced group 2 innate lymphoid cells acquire memory-like properties and enhance allergic lung inflammation. *Immunity*. 2016;45(1):198-208.
8. Rajput C, Han M, Ishikawa T, et al. Early-life heterologous rhinovirus infections induce an exaggerated asthma-like phenotype. *J Allergy Clin Immunol*. 2020;146(3):571-82 e3.
9. Neeland MR, Novakovic B, Dang TD, Perrett KP, Koplin JJ, Saffery R. Hyper-inflammatory monocyte activation following endotoxin exposure in food allergic infants. *Front Immunol*. 2020;11:567981.
10. Pasquali C, Salami O, Taneja M, et al. Enhanced mucosal antibody production and protection against respiratory infections following an orally administered bacterial extract. *Front Med (Lausanne)*. 2014;1:41.
11. Brandi P, Conejero L, Cueto FJ, et al. Trained immunity induction by the inactivated mucosal vaccine MV130 protects against experimental viral respiratory infections. *Cell Rep*. 2022;38(1):110184.

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

12. Eljaszewicz A, Ruchti F, Radzikowska U, et al. Trained immunity and tolerance in innate lymphoid cells, monocytes, and dendritic cells during allergen-specific immunotherapy. *J Allergy Clin Immunol*. 2021;147(5):1865-1877.
13. Benito-Villalvilla C, Perez-Diego M, Angelina A, et al. Allergoid-mannan conjugates reprogram monocytes into tolerogenic dendritic cells via epigenetic and metabolic rewiring. *J Allergy Clin Immunol*. 2022;149(1):212-222. e9.
14. Quintin J, Saeed S, Martens JHA, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe*. 2012;12(2):223-232.
15. Kleinnijenhuis J, Quintin J, Preijers F, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A*. 2012;109(43):17537-17542.
16. Divangahi M, Aaby P, Khader SA, et al. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. *Nat Immunol*. 2021;22(1):2-6.
17. Naik S, Larsen SB, Gomez NC, et al. Inflammatory memory sensitizes skin epithelial stem cells to tissue damage. *Nature*. 2017;550(7677):475-480.
18. Kaufmann E, Sanz J, Dunn JL, et al. BCG educates hematopoietic stem cells to generate protective innate immunity against tuberculosis. *Cell*. 2018;172(1–2):176-190. e19.
19. Chavakis T, Mitroulis I, Hajishengallis G. Hematopoietic progenitor cells as integrative hubs for adaptation to and fine-tuning of inflammation. *Nat Immunol*. 2019;20(7):802-811.
20. Dominguez-Andres J, Netea MG. Long-term reprogramming of the innate immune system. *J Leukoc Biol*. 2019;105(2):329-338.
21. Dominguez-Andres J, Arts RJW, Bekkering S, et al. In vitro induction of trained immunity in adherent human monocytes. *STAR Protoc*. 2021;2(1):100365.
22. Fanucchi S, Dominguez-Andres J, Joosten LAB, Netea MG, Mhlanga MM. The intersection of epigenetics and metabolism in trained immunity. *Immunity*. 2021;54(1):32-43.
23. Dominguez-Andres J, Novakovic B, Li Y, et al. The itaconate pathway is a central regulatory node linking innate immune tolerance and trained immunity. *Cell Metab*. 2019;29(1):211-20 e5.
24. Arts RJ, Novakovic B, Ter Horst R, et al. Glutaminolysis and fumarate accumulation integrate Immunometabolic and epigenetic programs in trained immunity. *Cell Metab*. 2016;24(6):807-819.
25. Cheng SC, Quintin J, Cramer RA, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science*. 2014;345(6204):1250684.
26. Dominguez-Andres J, Fanucchi S, Joosten LAB, Mhlanga MM, Netea MG. Advances in understanding molecular regulation of innate immune memory. *Curr Opin Cell Biol*. 2020;63:68-75.
27. Ebihara T, Tatematsu M, Fuchimukai A, et al. Trained innate lymphoid cells in allergic diseases. *Allergol Int*. 2021;70(2):174-180.
28. Quinn SM, Cunningham K, Raverdeau M, et al. Anti-inflammatory trained immunity mediated by helminth products attenuates the induction of T cell-mediated autoimmune disease. *Front Immunol*. 2019;10:1109.
29. Gyssens IC, Netea MG. Heterologous effects of vaccination and trained immunity. *Clin Microbiol Infect*. 2019;25(12):1457-1458.
30. Benn CS, Fisker AB, Rieckmann A, Sorup S, Aaby P. Vaccinology: time to change the paradigm? *Lancet Infect Dis*. 2020;20(10):e274-e283.
31. Boyd SD, Jackson KJL. Predicting vaccine responsiveness. *Cell Host Microbe*. 2015;17(3):301-307.
32. de Bree LCJ, Koeken V, Joosten LAB, et al. Non-specific effects of vaccines: current evidence and potential implications. *Semin Immunol*. 2018;39:35-43.
33. Kleinnijenhuis J, Quintin J, Preijers F, et al. Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J Innate Immun*. 2014;6(2):152-158.
34. Arts RJW, Moorlag S, Novakovic B, et al. BCG vaccination protects against experimental viral infection in humans through the induction of cytokines associated with trained immunity. *Cell Host Microbe*. 2018;23(1):89-100 e5.
35. Kleinnijenhuis J, Quintin J, Preijers F, et al. BCG-induced trained immunity in NK cells: role for non-specific protection to infection. *Clin Immunol*. 2014;155(2):213-219.
36. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis*. 2011;204(2):245-252.
37. Biering-Sorensen S, Aaby P, Lund N, et al. Early BCG-Denmark and neonatal mortality among infants weighing <2500 g: a randomized controlled trial. *Clin Infect Dis*. 2017;65(7):1183-1190.
38. Giamarellos-Bourboulis EJ, Tsilika M, Moorlag S, et al. Activate: randomized clinical trial of BCG vaccination against infection in the elderly. *Cell*. 2020;183(2):315-23 e9.
39. Brueggeman JM, Zhao J, Schank M, Yao ZQ, Moorman JP. Trained immunity: an overview and the impact on COVID-19. *Front Immunol*. 2022;13:837524.
40. Geckin B, Konstantin Fohse F, Dominguez-Andres J, Netea MG. Trained immunity: implications for vaccination. *Curr Opin Immunol*. 2022;77:102190.
41. Ovsyannikova IG, Reid KC, Jacobson RM, Oberg AL, Klee GG, Poland GA. Cytokine production patterns and antibody response to measles vaccine. *Vaccine*. 2003;21(25–26):3946-3953.
42. Nielsen S, Sujan HM, Benn CS, Aaby P, Hanifi SMA. Oral polio vaccine campaigns may reduce the risk of death from respiratory infections. *Vaccines (Basel)*. 2021;9(10):1133.
43. Gillard GO, Bivas-Benita M, Hovav AH, et al. Thy1+ NK [corrected] cells from vaccinia virus-primed mice confer protection against vaccinia virus challenge in the absence of adaptive lymphocytes. *PLoS Pathog*. 2011;7(8):e1002141.
44. Piedra PA, Gaglani MJ, Kozinetz CA, et al. Trivalent live attenuated intranasal influenza vaccine administered during the 2003-2004 influenza type a (H3N2) outbreak provided immediate, direct, and indirect protection in children. *Pediatrics*. 2007;120(3):e553-e564.
45. Lee YJ, Lee JY, Jang YH, Seo SU, Chang J, Seong BL. Non-specific effect of vaccines: immediate protection against respiratory syncytial virus infection by a live attenuated influenza vaccine. *Front Microbiol*. 2018;9:83.
46. Razi CH, Harmanci K, Abaci A, et al. The immunostimulant OM-85 BV prevents wheezing attacks in preschool children. *J Allergy Clin Immunol*. 2010;126(4):763-769.
47. Koatz AM, Coe NA, Ciceran A, Alter AJ. Clinical and immunological benefits of OM-85 bacterial lysate in patients with allergic rhinitis, asthma, and COPD and recurrent respiratory infections. *Lung*. 2016;194(4):687-697.
48. Alecsandru D, Valor L, Sanchez-Ramon S, et al. Sublingual therapeutic immunization with a polyvalent bacterial preparation in patients with recurrent respiratory infections: immunomodulatory effect on antigen-specific memory CD4+ T cells and impact on clinical outcome. *Clin Exp Immunol*. 2011;164(1):100-107.
49. Garcia Gonzalez LA, Arrutia DF. Mucosal bacterial immunotherapy with MV130 highly reduces the need of tonsillectomy in adults with recurrent tonsillitis. *Hum Vaccin Immunother*. 2019;15(9):2150-2153.
50. Nieto A, Mazon A, Nieto M, et al. Bacterial mucosal immunotherapy with MV130 prevents recurrent wheezing in children: a randomized, double-blind, placebo-controlled clinical trial. *Am J Respir Crit Care Med*. 2021;204(4):462-472.
51. Yang B, Foley S. First experience in the UK of treating women with recurrent urinary tract infections with the bacterial vaccine Uromune((R)). *BJU Int*. 2018;121(2):289-292.
52. Lorenzo-Gómez M-F, Foley S, Nickel JC, et al. Sublingual MV140 for prevention of recurrent urinary tract infections. *NEJM Evidence*. 2022;1(4):EVIDoa2100018.

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

53. Benito-Villalvilla C, Cirauqui C, Diez-Rivero CM, Casanovas M, Subiza JL, Palomares O. MV140, a sublingual polyvalent bacterial preparation to treat recurrent urinary tract infections, licenses human dendritic cells for generating Th1, Th17, and IL-10 responses via Syk and MyD88. *Mucosal Immunol*. 2017;10(4):924-935.
54. Martin-Cruz L, Sevilla-Ortega C, Benito-Villalvilla C, et al. A combination of Polybacterial MV140 and Candida albicans V132 as a potential novel trained immunity-based vaccine for genitourinary tract infections. *Front Immunol*. 2020;11:612269.
55. Guallar-Garrido S, Julian E. Bacillus Calmette-Guerin (BCG) therapy for bladder cancer: an update. *Immunotargets Ther*. 2020;9:1-11.
56. Ristori G, Faustman D, Matarese G, Romano S, Salvetti M. Bridging the gap between vaccination with Bacille Calmette-Guerin (BCG) and immunological tolerance: the cases of type 1 diabetes and multiple sclerosis. *Curr Opin Immunol*. 2018;55:89-96.
57. Ristori G, Buzzi MG, Sabatini U, et al. Use of Bacille Calmette-Guerin (BCG) in multiple sclerosis. *Neurology*. 1999;53(7):1588-1589.
58. Ristori G, Romano S, Cannoni S, et al. Effects of Bacille Calmette-Guerin after the first demyelinating event in the CNS. *Neurology*. 2014;82(1):41-48.
59. La Rocca C, Carbone F, De Rosa V, et al. Immunometabolic profiling of T cells from patients with relapsing-remitting multiple sclerosis reveals an impairment in glycolysis and mitochondrial respiration. *Metabolism*. 2017;77:39-46.
60. Kuhtreiber WM, Tran L, Kim T, et al. Long-term reduction in hyperglycemia in advanced type 1 diabetes: the value of induced aerobic glycolysis with BCG vaccinations. *NPJ Vaccines*. 2018;3:23.
61. Foster SL, Hargreaves DC, Medzhitov R. Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature*. 2007;447(7147):972-978.
62. Agache I, Annesi-Maesano I, Bonertz A, et al. Prioritizing research challenges and funding for allergy and asthma and the need for translational research-the European strategic forum on allergic diseases. *Allergy*. 2019;74(11):2064-2076.
63. Boyle RJ, Shamji MH. Developments in the field of allergy in 2020 through the eyes of clinical and experimental allergy. *Clin Exp Allergy*. 2021;51(12):1531-1537.
64. Akdis CA, Arkwright PD, Bruggen MC, et al. Type 2 immunity in the skin and lungs. *Allergy*. 2020;75(7):1582-1605.
65. Palomares O, Akdis M, Martin-Fontecha M, Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunol Rev*. 2017;278(1):219-236.
66. Palomares O, Sanchez-Ramon S, Davila I, et al. dIvergEnt: how IgE Axis contributes to the continuum of allergic asthma and anti-IgE therapies. *Int J Mol Sci*. 2017;18(6):1328.
67. Cecchi L, D'Amato G, Annesi-Maesano I. External exposome and allergic respiratory and skin diseases. *J Allergy Clin Immunol*. 2018;141(3):846-857.
68. Agache I, Sampath V, Aguilera J, et al. Climate change and global health: a call to more research and more action. *Allergy*. 2022;77(5):1389-1407.
69. Alashkar Alhamwe B, Alhamdan F, Ruhl A, Potaczek DP, Renz H. The role of epigenetics in allergy and asthma development. *Curr Opin Allergy Clin Immunol*. 2020;20(1):48-55.
70. Agache I, Cojanu C, Laculiceanu A, Rogozea L. Genetics and epigenetics of allergy. *Curr Opin Allergy Clin Immunol*. 2020;20(3):223-232.
71. Agache I, Akdis CA, Akdis M, et al. EAACI biologicals guidelines-recommendations for severe asthma. *Allergy*. 2021;76(1):14-44.
72. Hassoun D, Malard O, Barbarot S, Magnan A, Colas L. Type 2 immunity-driven diseases: towards a multidisciplinary approach. *Clin Exp Allergy*. 2021;51(12):1538-1552.
73. Feleszko W, Jartti T, Bacharier LB. Current strategies for phenotyping and managing asthma in preschool children. *Curr Opin Allergy Clin Immunol*. 2022;22(2):107-114.
74. Esposito S, Soto-Martinez ME, Feleszko W, Jones MH, Shen KL, Schaad UB. Nonspecific immunomodulators for recurrent respiratory tract infections, wheezing and asthma in children: a systematic review of mechanistic and clinical evidence. *Curr Opin Allergy Clin Immunol*. 2018;18(3):198-209.
75. Lauzon-Joset JF, Mincham KT, Scott NM, et al. Protection against neonatal respiratory viral infection via maternal treatment during pregnancy with the benign immune training agent OM-85. *Clin Transl Immunol*. 2021;10(7):e1303.
76. Troy NM, Strickland D, Serralha M, et al. Protection against severe infant lower respiratory tract infections by immune training: mechanistic studies. *J Allergy Clin Immunol*. 2022;150(1):93-103.
77. Pivniouk V, Gimenes-Junior JA, Ezeh P, et al. Airway administration of OM-85, a bacterial lysate, blocks experimental asthma by targeting dendritic cells and the epithelium/IL-33/ILC2 axis. *J Allergy Clin Immunol*. 2022;149(3):943-956.
78. Cirauqui C, Benito-Villalvilla C, Sanchez-Ramon S, et al. Human dendritic cells activated with MV130 induce Th1, Th17 and IL-10 responses via RIPK2 and MyD88 signalling pathways. *Eur J Immunol*. 2018;48(1):180-193.
79. Machiels B, Dourcy M, Xiao X, et al. A gammaherpesvirus provides protection against allergic asthma by inducing the replacement of resident alveolar macrophages with regulatory monocytes. *Nat Immunol*. 2017;18(12):1310-1320.
80. Steer CA, Matha L, Shim H, Takei F. Lung group 2 innate lymphoid cells are trained by endogenous IL-33 in the neonatal period. *JCI Insight*. 2020;5(14):e135961.
81. Hurrell BP, Shafiei Jahani P, Akbari O. Social networking of group two innate lymphoid cells in allergy and asthma. *Front Immunol*. 2018;9:2694.
82. Matha L, Martinez-Gonzalez I, Steer CA, Takei F. The fate of activated group 2 innate lymphoid cells. *Front Immunol*. 2021;12:671966.
83. Yu W, Freeland DMH, Nadeau KC. Food allergy: immune mechanisms, diagnosis and immunotherapy. *Nat Rev Immunol*. 2016;16(12):751-765.
84. Sicherer SH, Warren CM, Dant C, Gupta RS, Nadeau KC. Food allergy from infancy through adulthood. *J Allergy Clin Immunol Pract*. 2020;8(6):1854-1864.
85. Tulic MK, Hodder M, Forsberg A, et al. Differences in innate immune function between allergic and nonallergic children: new insights into immune ontogeny. *J Allergy Clin Immunol*. 2011;127(2):470-478. e1.
86. Zhang Y, Collier F, Naselli G, et al. Cord blood monocyte-derived inflammatory cytokines suppress IL-2 and induce nonclassic "T(H)2-type" immunity associated with development of food allergy. *Sci Transl Med*. 2016;8(321):321ra8.
87. Neeland MR, Koplin JJ, Dang TD, et al. Early life innate immune signatures of persistent food allergy. *J Allergy Clin Immunol*. 2018;142(3):857-64 e3.
88. Neeland MR, Andorf S, Manohar M, et al. Mass cytometry reveals cellular fingerprint associated with IgE+ peanut tolerance and allergy in early life. *Nat Commun*. 2020;11(1):1091.
89. Neeland MR, Andorf S, Dang TD, et al. Altered immune cell profiles and impaired CD4 T-cell activation in single and multi-food allergic adolescents. *Clin Exp Allergy*. 2021;51(5):674-684.
90. Pfaar O, Creticos PS, Kleine-Tebbe J, Canonica GW, Palomares O, Schulke S. One hundred ten years of allergen immunotherapy: a broad look into the future. *J Allergy Clin Immunol Pract*. 2021;9(5):1791-1803.
91. Shamji MH, Sharif H, Layhadi JA, Zhu R, Kishore U, Renz H. Diverse immune mechanisms of allergen immunotherapy for allergic rhinitis with and without asthma. *J Allergy Clin Immunol*. 2022;149(3):791-801.
92. Sharif H, Acharya S, Dhondalay GKR, et al. Altered chromatin landscape in circulating T follicular helper and regulatory cells following grass pollen subcutaneous and sublingual immunotherapy. *J Allergy Clin Immunol*. 2021;147(2):663-676.
93. Golebski K, Layhadi JA, Sahiner U, et al. Induction of IL-10-producing type 2 innate lymphoid cells by allergen immunotherapy is associated with clinical response. *Immunity*. 2021;54(2):291-307. e7.

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

94. Morita H, Kubo T, Ruckert B, et al. Induction of human regulatory innate lymphoid cells from group 2 innate lymphoid cells by retinoic acid. *J Allergy Clin Immunol*. 2019;143(6):2190-2201. e9.
95. Sirvent S, Soria I, Cirauqui C, et al. Novel vaccines targeting dendritic cells by coupling allergoids to nonoxidized mannan enhance allergen uptake and induce functional regulatory T cells through programmed death ligand 1. *J Allergy Clin Immunol*. 2016;138(2):558-567. e11.

How to cite this article: Martín-Cruz L, Sevilla-Ortega C, Angelina A, et al. From trained immunity in allergy to trained immunity-based allergen vaccines. *Clin Exp Allergy*. 2022;00:1-11. doi:10.1111/cea.14261

13652222, 0, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/cea.14261 by Spanish Cochrane National Provision (Ministerio de Sanidad), Wiley Online Library on [10/12/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Current Allergy and Asthma Reports

C-type Lectin Receptor Mediated Modulation of T2 Immune Responses to Allergens

--Manuscript Draft--

Manuscript Number:	ALAS-D-22-00051
Full Title:	C-type Lectin Receptor Mediated Modulation of T2 Immune Responses to Allergens
Article Type:	Review
Section/Category:	Allergens
Corresponding Author:	Oscar Palomares Complutense University of Madrid: Universidad Complutense de Madrid Madrid, 28040 SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Complutense University of Madrid: Universidad Complutense de Madrid
Corresponding Author's Secondary Institution:	
First Author:	Alba Angelina
First Author Secondary Information:	
Order of Authors:	Alba Angelina
	Leticia Martín-Cruz
	Andrés de la Rocha-Muñoz
	Begoña Lavín-Plaza
	Oscar Palomares
Order of Authors Secondary Information:	
Funding Information:	
Abstract:	Purpose of review. Allergic diseases represent a major health problem of increasing prevalence worldwide. In allergy, dendritic cells (DCs) contribute both the pathophysiology and the induction of healthy immune responses to the allergens. Different studies have reported that some common allergens contain glycans in their structure. C-type lectin receptors (CLRs) expressed by DCs recognize carbohydrate structures and are crucial in allergen uptake, presentation, and polarization of T cell responses. This review summarizes the recent literature regarding the role of CLRs in the regulation of type 2 immune responses to allergens. Recent findings. In this review, we highlight the capacity of CLRs to recognize carbohydrates in common allergens triggering different signaling pathways involved in the polarization of CD4+ T cells towards specific Th2 responses. Under certain conditions, specific CLRs could also promote tolerogenic responses to allergens, which it might well be exploited to develop novel therapeutic approaches of allergen-specific immunotherapy (AIT), the singly treatment with potential disease-modifying capacity for allergic disease. At this regard, polymerized allergens conjugated to non-oxidized mannan (allergoid-mannan conjugated) are next generation vaccines targeting DCs via CLRs that promote regulatory T cells, thus favouring allergen tolerance both in preclinical models and clinical trials. Summary. A better understanding of the role of CLRs in the development of allergy and in the induction of allergen tolerance might well pave the way for the design of novel strategies for allergic diseases.

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript
Click here to access/download;Manuscript;Review CLRs_17.11.22_OsP.docx
Click here to view linked References

TITLE PAGE

C-type Lectin Receptor Mediated Modulation of T2 Immune Responses to Allergens

Alba Angelina[1#], Leticia Martín-Cruz[1#], Andrés de la Rocha-Muñoz[1,2], Begoña Lavín-Plaza[1], Oscar Palomares[1*]

[1]Department of Biochemistry and Molecular Biology, School of Chemistry, Complutense University of Madrid, Madrid, Spain
[2] Autonomous University of Madrid, Spain

# Contributed equally

* **Corresponding Author:** Oscar Palomares, PhD
Department of Biochemistry and Molecular Biology,
School of Chemistry, Complutense University of Madrid,
Avenida Complutense s/n,
28040 Madrid, Spain
Telephone: + 34 913944159
Email: oscar.palomares@quim.ucm.es

Keywords: C-type lectin receptors (CLRs), type-2 immune responses, allergy, allergens, allergen-specific immunotherapy (AIT), mannan.

Abstract

Purpose of review. Allergic diseases represent a major health problem of increasing prevalence worldwide. In allergy, dendritic cells (DCs) contribute both the pathophysiology and the induction of healthy immune responses to the allergens. Different studies have reported that some common allergens contain glycans in their structure. C-type lectin receptors (CLRs) expressed by DCs recognize carbohydrate structures and are crucial in allergen uptake, presentation, and polarization of T cell responses. This review summarizes the recent literature regarding the role of CLRs in the regulation of type 2 immune responses to allergens.

Recent findings. In this review, we highlight the capacity of CLRs to recognize carbohydrates in common allergens triggering different signaling pathways involved in the polarization of $CD4^+$ T cells towards specific Th2 responses. Under certain conditions, specific CLRs could also promote tolerogenic responses to allergens, which it might well be exploited to develop novel therapeutic approaches of allergen-specific immunotherapy (AIT), the singly treatment with potential disease-modifying capacity for allergic disease. At this regard, polymerized allergens conjugated to non-oxidized mannan (allergoid-mannan conjugated) are next generation vaccines targeting DCs via CLRs that promote regulatory T cells, thus favouring allergen tolerance both in preclinical models and clinical trials.

Summary. A better understanding of the role of CLRs in the development of allergy and in the induction of allergen tolerance might well pave the way for the design of novel strategies for allergic diseases.

Introduction

Our immune system orchestrate type 2 immune responses to protect us against helminths, venoms and toxins. However, under certain conditions aberrant type 2 immune responses arise leading to different type 2 inflammatory disorders such as allergic diseases. Allergy represents a global health problem of increasing prevalence that affect around 30% of the population worldwide [1, 2]. Type 2-mediated allergic diseases include allergic rhinitis, allergic asthma, atopic dermatitis, food allergy and anaphylaxis [2]. Dendritic cells (DCs) are professional antigen-presenting cells essential for the generation of proper immune responses [3]. In the context of allergy, DCs recognize and internalize encountered allergens within the skin, respiratory and gastrointestinal tracts, which are processed and then presented to naïve $CD4^+$ T cells in the lymph nodes. Depending on the antigens and the signals that DCs receive in the peripheral tissues and in the lymph nodes, antigen presentation might trigger the induction of potent allergen-specific Th2 responses or regulatory T (Treg) cells, thus promoting allergic inflammation or allergen tolerance, respectively [4-6]. DCs are equipped with a large number of pattern-recognition receptors (PRRs) including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) among others [7, 8]. CLRs recognize a wide range of carbohydrate structures contained in allergens, which might influence allergen uptake, subsequent processing and presentation as well as specific T cell polarization [4, 9]. The final functional outcomes not only depend on the specific targeted CLRs but also on the physical nature, affinity and avidity of the carbohydrate ligands [7]. A quick expansion in the understanding of the role of CLRs in the context of allergy has taken place over the last years [10]. Some allergens from common allergenic sources such as house dust mite (HDM), peanut or animal dander contain glycan structures recognized by specific CLRs, which in turn promote allergic responses [11]. On the other hand, compelling experimental evidence also demonstrated that triggering of specific CLRs might induce immune regulation and allergen tolerance [12]. At this

regard, allergoids conjugated to mannan are next generation vaccines for allergen-specific immunotherapy (AIT) targeting DCs that are able to induce allergen tolerance [12, 13]. Allergoid-mannan conjugates have demonstrated efficacy and safety in phase II trials and are currently under evaluation in phase III clinical trials [14]. Herein, we review and provide an update on the role of CLRs in the regulation of type 2 immune responses to allergens and discuss the most relevant findings related to the potential therapeutic applications of CLRs ligands for treatment of allergic diseases.

Structural and functional feature of C-type Lectin Receptors

The C-type Lectin Receptors (CLRs) are a superfamily of receptors characterized by the presence of at least one C-type lectin-like domain (CTLD), which is defined by its ability to bind carbohydrates in a calcium-dependent manner [15]. The CLRs superfamily is formed by more than 1,000 members, organized in 17 groups, and recognize self and non-self ligands involved in different functions such as cell adhesion, phagocytosis, homeostasis, and innate and adaptive immunity [7, 16]. Ligand recognition occurs generally through their extracellular CTLD which is tightly regulated by specific amino acid motifs, calcium ions and the carbohydrate structure [17]. Based on the cytoplasmic signaling motifs and the signaling pathways triggered upon ligand binding, myeloid CLRs can be classified in three categories (**Figure 1**): CLRs coupled to the spleen tyrosine kinase (Syk), CLRs containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) and CLRs without immunoreceptor tyrosine-based activating motif (ITAM) or ITIM domains [16]. In this review, we will focus on membrane-associated CLRs that are mainly expressed in myeloid cells (**Table 1**) and specifically on dectin-1, dectin-2, DC immunoreceptor (DCIR), mannose receptor (MR) and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) as CLRs involved in the regulation of type 2 immune responses to allergens [10] (**Figure 1**).

Dectin-1

Dectin-1 is a type II transmembrane protein classified as HemITAM-based CLRs as it contains half of the classical ITAM (YXXL tandem repeats), in their intercellular tail allowing direct binding to Syk (**Figure 1**) [16]. Dectin-1 was described to bind β-glucans, commonly found in the cell wall of plants, fungi, and some bacteria. Other ligands such as galectin-9, annexins or tropomyosin, among others, have been also identified, suggesting dectin-1 as one of the most versatile receptors in myeloid cells [18]. The protein surface residues Trp221 and His223 are key in the ligand binding in a calcium independent manner [19]. Dectin-1 is expressed in DCs, monocytes, macrophages, neutrophils, B cells and the Gr-1$^+$ subset of splenic T cells in mice (**Table 1**) [10]. Similar expression pattern has been described for human, including expression in eosinophils and mast cells [19]. Dectin-1 displays a single CTLD connected by a stalk to a single transmembrane region, and a cytoplasmic tail with the hemITAM (**Figure 1**). Phosphorylation of the tyrosine residue generates docking sites for the SH2 Syk domains, triggering a conformational change and activation through autophosphorylation [16]. Syk can phosphorylate several molecules inducing a plethora of molecular pathways involved in proinflammatory responses, such as cytokine production, reactive oxygen species (ROS) generation, phagocytosis, and trained immunity [16, 18, 20].

Dectin-2

Dectin-2 is a type II transmembrane protein classified as ITAM-coupled CLRs [16]. Dectin-2 has high affinity for α-mannan glycan structures and binding occurs in a calcium-dependent fashion [10]. Moreover, dectin-2 has been shown to have a putative endogenous ligand on CD4$^+$ T cells and to recognize the HDM allergens [17]. Dectin-2 is expressed in macrophages and some DC subsets in both mouse and human (**Table 1**) [10]. Dectin-2 is formed by a single extracellular CTLD connected by a neck region to a short cytoplasmic tail that lacks a clear intracellular signaling motif [17]. Association of dectin-2 with adaptor proteins such as, the Fc receptor γ (FcRγ) chain or DNAX-

activation protein 12 (DAP12), that contain ITAM, is required for the engagement to Syk (**Figure 1**) [16]. Similar to dectin-1, several signaling pathways are induced via dectin-2, highlighting the expression of cysteinyl leukotrienes, proinflammatory lipid mediators and the Th17 polarizing cytokines IL-1β and IL-23 in DCs [16, 21].

DCIR

DCIR is a type II transmembrane protein classified as an ITIM-containing CLR and shows specificity for mannose- and fucose-based glycans and its binding is modulated by the glycosylation of the CTLD [22]. DCIR is expressed in human monocytes, macrophages, granulocytes, B cells and DCs (**Table 1**) [16]. Four homologs have been described in mice, highlighting Dcir1 which is expressed in B cells, monocytes/macrophages and DCs [16]. DCIR structure has a single CTLD followed by a neck and transmembrane domains that connect with the cytoplasmic tail that has an ITIM which upon phosphorylation of the tyrosine residue transduces inhibitory signals by activating phosphatases SHP-1 and SHP-2 [23]. DCIR inhibits nuclear factor-κB (NF-κB) signaling, limit DC expansion and activates the STAT1-type I IFN signaling, controlling differentiation of T lymphocytes towards Th1 [16, 24].

Mannose receptor and DC-SIGN

Mannose receptor (MR) was one of the first CLRs discovered. MR is a type I integral transmembrane glycoprotein that recognizes glycans such as mannose- and fucose-based structures, N-acetylglucosamine and sulfated glycan structures in a calcium dependent manner [10, 16, 17]. MR expression was originally restricted to resident macrophages, but now it is known to be also expressed in monocyte-derived DCs, lymphatic and hepatic epithelium, kidney mesangial cells, tracheal smooth muscle cells and retinal pigment epithelium (**Table 1**) [16, 19]. MR has three regions, a cysteine-rich domain, a fibronectin type II-like domain, that binds collagen; and 8 CTLDs, which forms the extracellular part, followed by a transmembrane region and a cytoplasmic tail that is

involved in receptor internalization and recycling (**Figure 1**) [17]. Importantly, like DC-SIGN, MR lacks ITAM or ITIM [17]. MR has been implicated in pathogen phagocytosis via the activation of the complex Rac-1, Cdc42 and PAK-1 [17]. Activation of MR promote anti-inflammatory properties in immature monocyte-derived DCs by inducing IL-10 and IL-1Ra production [16]. However, MR-deficient macrophages produce less TNFα and more IL-10 [16]. These differences might strongly depend on the nature, affinity and avidity of ligand binding, but further research is still needed to better clarify the induced signaling pathways.

DC-SIGN (CD209) is a type II transmembrane protein and binds, in a calcium-dependent manner, a broad spectrum of mannose and fucose ligands found in viruses, parasites, fungi, bacteria, and self-ligands such as intercellular adhesion molecule (ICAM)-2, ICAM-3 and Mac-1 [17]. It is expressed in immature and mature monocyte-derived DCs, dermal DCs and macrophages (**Table 1**) [10]. DC-SIGN is arranged as a tetramer with autonomous CTLD that interact through and α-helix neck domain, followed by a transmembrane region and a cytoplasmic domain [15]. The signaling cascade affected by DC-SIGN depends on the type of ligand that binds. DC-SIGN do not induce an immediate downstream signaling, however, it has been shown to modulate the response initiated by the TLRs [17]. In addition, an intracellular signalosome can be formed inducing, on one side, anti-inflammatory cytokines such as IL-10 and suppressing, on the other side, pro-inflammatory responses via acetylation of the NF-κB subunit p65 and production of IL-6 and TNFα (**Figure 1**) [17].

The role of CLR in the context of allergic diseases

Allergic diseases are mainly type 2 immune-mediated disorders characterized by the formation of specific IgE antibodies against innocuous substances called allergens. Allergic sensitization occurs after the first contact with the allergen, which is captured by

DCs in the airways, gut, or skin. DCs process and transport the allergen to the drain lymph nodes where it is presented to naïve $CD4^+$ T cells, leading the generation of allergen specific $CD4^+$ Th2 cells. After IgE class-switching, B cells produce high amounts of allergen-specific IgE that bind to the high-affinity FcεRI on mast cells and basophils, thus leading to patient's sensitization [2, 25]. New allergen encounters trigger cross-linking of the IgE-FcεRI complexes on mast cells and basophils, inducing the release of the anaphylactogenic mediators that are responsible for the immediate clinical symptoms. Memory allergen-specific Th2 cells activated via IgE-facilitated allergen presentation by DCs and B cells and type 2 innate lymphoid cells (ILC2) activated by epithelial cell-derived alarmins (TSLP, IL-33 and IL-25) produce large amounts of IL-4, IL-5, IL-9 and IL-13 contributing to maintain allergen specific-IgE levels, eosinophilia, mucus production, recruitment of inflammatory cell and tissue inflammation, which are involved in the chronicity and the most severe clinical manifestations of allergy [2, 25].

The initiation of type 2 immune response prompted by allergens entails its uptake and processing by antigen presenting cells, mainly DCs, a process that might be enhanced by specific CLRs. Common allergens such as Der p 1, Der p 2, Fel d 1, Ara h 1, Bla g 2 or Can f 1 are glycoproteins, many of which contain structurally-related oligosaccharides. Compelling experimental evidence show that the common oligosaccharide structure on allergens is a pentasaccharide core, Man3-GlcNAc2, which often contains additional monosaccharides [11, 26]. CLRs, including dectin-1, dectin-2, DCIR, MR and DC-SIGN express on DCs and other myeloid immune cells can recognize carbohydrate structures on allergens, enhancing their uptake and also triggering different downstream signalling responses that might well promote type 2-mediated responses or tolerance depending on the specific CLRs engaged and the subsequent signalling pathways initiated, the physical nature of the carbohydrate ligands as well as on the affinity and avidity of the carbohydrate-CLR interaction [7, 27]. In the next paragraphs, we will summarize the most recent findings related to the role of specific CLRs that have

been involved in the recognition of different allergens and how this might regulate the initiation and maintenance of type 2 immune responses in the context of allergic diseases.

There are conflicting data about the role of dectin-1 in allergy. Murine models of HDM- and ovalbumin-induced airway inflammation have demonstrated that dectin-1 deficient (Clec7a$^{-/-}$) mice displayed less eosinophilic and neutrophilic inflammation as well as Th2 and Th17 cells compared with wild-type mice [28, 29]. Dectin-1 expressed by CD11b^{+} DCs sense some glycans in the HDM extract and promotes allergic airway inflammation [28] (**Figure 2**). Similarly, dectin-1 signaling has been proven to be essential for the development of Japanese cedar pollen-induced allergic rhinitis in mice [30] and participates in lung inflammation in a murine model of fungal allergy through the induction of IL-22 [31]. By contrast, it has been demonstrated that invertebrate tropomyosin (for example, Der p 10 in HDM) binds to dectin-1 at the mucosal surfaces and mediates protection against allergic diseases [32]. In addition, it has been demonstrated that dectin-1 is downregulated in the epithelium of allergic patients due to the aberrant production of IL-33 [33]. In line with these results, Gu *et al* have described that dectin-1 activation suppressed allergic type 2 responses [34]. Although many research effort have been made in understanding the role of dectin-1 in the promotion or prevention of type 2 immune responses to allergens, further studies are still needed to fully elucidate its actual role in allergic diseases.

Dectin-2 is also involved in HDM-induced allergic airway inflammation as shown different mouse models [35-37]. Notably, dectin-2-deficient mice display decreased eosinophilic and neutrophilic inflammation and Th2 responses in lung after HDM-induced sensitization [35]. Blocking of dectin-2 with specific antibodies inhibits HDM- as well as Der p 2-induced IL-5 and IL-13 production in cocultures of DC-T cells from asthmatic patients [38], which are characterized by an increased dectin-2 expression profile in peripheral blood mononuclear cells [39] (**Figure 2**).

Although DCIR is an inhibitory CLR, it has been involved in allergen processing and signaling. DCIR binds cockroach allergen Bla g 2 and mediates its uptake by human basophils and mast cells, leading their activation and contributing to Th2 responses [40, 41]. In a mice model of atopic dermatitis, DCIR (*DCIR* $^{-/-}$) deficient mice exposed to Bla g 2 display an attenuation of allergen-induced skin inflammation compared to wild-type mice [41], suggesting that DCIR might well play a key role during skin sensitization and allergen-mediated inflammation in the context of atopic dermatitis.

MR has been involved in the recognition and internalization of different allergens from HDM (Der p 1 and Der p 2), dog (Can f 1), cat (Fel d 1) cockroach (Bla g 2) and peanut (Ara h 1, Ara h 3) through their carbohydrate moieties [42-45]. MR-deficient DCs show a reduced capacity to capture these allergens, although other receptors could be also implicated (**Figure 2**). Binding assays reveal that CTLD4-7 domains of MR mediate the binding of Der p 1, Der p 2, Can f 1, Ara h 1 and Bla g 2 [42]. However, Fel d 1 is a ligand of the MR cysteine-rich domain [45]. MR participates in the Th2 polarization imprinted by Der p 1 in DCs [42]. Similarly, a mice model of allergic rhinitis has showed that MR has an essential role in Th2 polarization induced by the airborne allergen Fel d 1 [45]. Interestingly, DCs from allergic patients express higher levels of MR and uptake Der p 1 more efficiently than DCs from healthy donors [43, 46]. On the other hand, MR-deficient lung macrophages have displayed reduced cockroach allergen uptake, and MR-deficient mice have showed increased lung inflammation and exacerbated Th2 responses, suggesting that MR might well also play a very important role in the attenuation of type 2 inflammatory responses to allergens in the airways [47]. Therefore, deeper investigations are still required to firmly elucidate the actual role of MR in the regulation of type 2 immune responses to allergens or tolerance induction within the context of different allergic diseases.

DC-SIGN has been also implicated in the immunopathology of allergy due to its capacity to recognize allergens from HDM (Der p 1), foods (Ara h 1), animal dander (Can

f 1, Fel d 1) and pollens (BG-60) [48, 49]. The major peanut allergen Ara h 1 binds to DC-SIGN activating DCs and inducing the polarization towards Th2 cells [50]. It has been described that peanut agglutinin is also a ligand of DC-SIGN that activates DCs *in vitro* [51]. Other common allergenic foods such as soybean, tree nuts, chicken egg and milk contain DC-SIGN-binding proteins, which have showed serum IgE-reactivity [51]. Allergens from HDM and chicken egg, Der p 2 and Gal d 2, are captured by human DCs through DC-SIGN binding and promote the priming of Th2 and Th22 responses [49] . The use of a DC-SIGN blocking antibody impair Der p 2 and Gal d 2 uptake by DCs as well as Th2 and Th22 polarization [49]. It has also been reported that HDM allergen Der p 7 can activate DCs via DC-SING binding and lead to polarization of Th2 *in vitro* [52] (**Figure 2**). Native or heat-inactivated allergens from HDM extract are endocytosed through DC-SIGN, reducing DC-SIGN expression on the surface of DCs, but inducing signaling events that promote the generation of Th2 cells [53]. However, it has been also shown that DC-SIGN deletion in DCs promoted Th2 polarization in autologous cocultures of Der p 1-stimulated DCs with T cells, suggesting that the axis Der p 1-DC-SIGN might well also promote allergen-specific Th1 or regulatory responses able to impair type 2 polarization [54]. Under this scenario, it was previously described that Der p 1 due to its cysteine protease activity can cleave DC-SIGN but not MR, which might play also an important role in the amplification of type 2 immune responses, thus enhancing its allergenicity [55, 56]. Therefore, further studies are also needed to clarify the actual role of DC-SIGN in the context of allergic diseases within the different potential scenarios.

Can we exploit CLRs as potential therapeutic targets for allergic diseases?

As above described, in addition to their role in the initiation of type 2 pro-inflammatory immune responses against encountered allergens, specific CLRs can also promote the generation of allergen-specific Th1 and/or Treg cells that might well contribute to the immunoregulation of allergic diseases and to the restoration of healthy immune responses to allergen. The final functional outcomes depend on the specific activated

CLRs and on the integration of the initiated downstream signalling pathways, which is also finely tuned by the physical nature of the carbohydrate ligands as well as by the affinity and avidity of the carbohydrate-CLRs engagement. Remarkably, it has been shown that depending on its 3D configuration, solubility and stoichiometry, the same carbohydrate structure might well simultaneously activate different CLRs within the same cell [7, 27]. The final integration of all these signals will determine the final functional outcomes that can be achieved in the context of allergic diseases. At this regard, different studies conducted so far have shown that activation of specific CLRs, such as dectin-1, DCIR, DC-SIGN or DEC205, induces anti-inflammatory responses that could alleviate allergic symptoms in patients [32, 57-60]. The potential immunoregulatory properties displayed by specific CLRs under certain conditions have been recently exploited to develop next generation vaccines for AIT [12].

AIT is the only treatment for allergic diseases with the potential capacity to induce long-lasting disease modification. AIT is based on repeated administration of high doses of the causative allergen to achieve a state of long-term tolerance that persists after treatment discontinuation [61, 62]. Although many clinical trials and real-life experience demonstrated AIT as a safe and effective treatment for many patients, it still faces important drawbacks in terms of efficacy, safety, the long duration of the treatment or the large number of administrations needed to induce tolerance, which is associated to low patient adherence [12, 61, 62]. In this regard, vaccines targeting DCs by coupling allergens to specific carbohydrate structures have been pointed as a suitable strategy that might well help to overcome these problems [10, 12]. This approach targets allergen vaccines to specific immune cells (i.e. DCs) thought specific CLRs, which might significantly increase the effective doses of the delivery allergens. Simultaneously, targeting specific CLRs with the conjugated carbohydrates elicit immunomodulatory responses in the same DCs, thus potentiating the capacity to induce allergen tolerance [10, 12]. Until now, the beneficial effects of several carbohydrates conjugated with

allergens have been reported for β-glucan, N-acetylgalactosamine, mannose and mannan [13, 63-70].

The most clinically advanced strategies for glycan-allergen conjugates for AIT are the next generation vaccines based on the use of polymerized allergens conjugated to non-oxidized mannan (allergoid-mannan conjugates).

As a proof of concept, initial preclinical studies demonstrated that allergoid-mannan conjugates from *P. pratense* grass pollen allergens targets DCs via CLRs (MR, DC-SIGN and dectin-2) significantly enhancing allergen uptake and promoting the generation of functional allergen-specific $FOXP3^+$ Treg cells by mechanisms partially depending on IL-10 and PD-L1 (**Figure 3**) [13, 71]. The tolerogenic properties imprinted by allergoid-mannan conjugates in DCs is driven by an enhanced production of ROS and a metabolic rewiring induced by mTOR signalling pathway consisting of an increased rate of glycolysis and lactate fermentation, which is impair by the presence of aluminium, a commonly used adjuvant for AIT [72]. Similarly, allergoid-mannan conjugates impairs the generation of pro-inflammatory and pro-allergic human macrophages, whereas potentiates the polarization of macrophages with regulatory functions [69]. In mice, after subcutaneous or sublingual immunization, allergoid-mannan conjugates showed better results than native allergens or allergoids in terms of increasing IgG2a/IgE, IFN-γ/IL-4 ratios, IL-10-producing cells and $FOXP3^+$ Treg cells [13, 71] (**Figure 3**). Remarkably, differentiation of macrophages and DCs from human monocytes in the presence of allergoid-mannan conjugates also induce tolerogenic profiles, as indicated by the high expression of the tolerogenic molecules IL-10, indoleamine 2,3-dioxygenase (IDO), PD-L1, suppressor of cytokine signalling (SOCS) 1, and SOCS3. In these freshly generated cells, lipopolysaccharide stimulation triggers a reduced release of the pro-inflammatory cytokines IL-6 and TNF-α, promotes an increase of the IL-10/TNF-α ratio and a greater capacity to induce functional $FOXP3^+$ Treg cells [68, 69]. The acquisition of this tolerogenic phenotype is supported by a metabolic and epigenetic reprogramming [68].

In this scenario, glucose metabolism is switched from Warburg effect and lactate production to mitochondrial oxidative phosphorylation. In addition, an epigenetic reprogramming within tolerogenic loci, lower expression levels of histone deacetylase genes, increased expression of the anti-inflammatory miRNA-146a/b and decreased proinflammatory miRNA-155 contributes to the induction of this phenotype [68].

Interestingly, in a phase II clinical trial enrolling 196 HDM-allergic patients it has been recently published that allergoid-mannan conjugates, either subcutaneously or sublingually administered, display efficacy and safety for the treatment of allergic rhinitis/rhinoconjunctivitis [14] (**Figure 3**). There are also ongoing other phase II clinical trials to assess the efficacy and safety of allergoid-mannan conjugates from grass (NCT02654223) and birch (EudraCT: 2018-002522-23 & 2020-004126-32) pollen for the treatment of allergic rhinitis/rhinoconjunctivitis, which results are expected to be published soon. In the same way, phase III clinical trials for allergoid-mannan conjugates are currently ongoing for the treatment of patients with mild to moderate HDM-induced asthma and rhinitis/rhinoconjunctivitis (NCT05400811) or birch pollen-induced allergic rhinitis/rhinoconjunctivitis (EudraCT: 2021-002252-36). Results from these ongoing clinical trials might well help to definitely determine if these next generation vaccines targeting CLRs display clinical benefits that can be translated into real-life improvements of allergic patients' quality of life.

Conclusion and future perspectives

Cellular and molecular mechanisms involved in the generation of type 2 immune responses to allergens has been deeply studied over the last years. Recent findings uncovered that allergens contain carbohydrate structures that are recognize by specific CLRs, which in turn activate different signaling pathways on the targeted cells, thus leading to the generation of allergen-specific $CD4^+$ Th2 cells. Under different conditions,

specific CLRs might well promote tolerogenic responses to allergens. A better knowledge on the actual carbohydrate fraction of allergens that interact with specific CLRs and how signaling pathways are orchestrated depending on the stoichiometry, affinity and avidity of this binding is still needed. Carbohydrate-modified allergens might represent the next generation vaccines targeting specific immune cells for AIT. At this regard, allergoid-mannan conjugates targeting to DCs enhance the allergen uptake and promote healthy immune response to allergens. Allergoid-mannan conjugated vaccines for grass pollen, birch pollen and HDM allergy have been already developed and assayed in phase II clinical trials and phase III clinical trials are currently ongoing. Overall, CLRs represent suitable targets for AIT but the better understanding of the mechanisms underlying the mode of action of superfamily of receptors at the molecular level in the context of allergic diseases might well open new opportunities for the development of future therapeutic approaches in the field of allergy.

Conflict of Interest

OP has received fee for lectures or participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, Diater, GSK, Pfizer, Inmunotek SL, Novartis, Sanofi Genzyme, Stallergenes and Regeneron. OP has received research grants from Inmunotek SL, Novartis SL, MINECO, MICINNIN and CAM. The rest of the authors declare no competing financial interests.

Human and Animal Rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

ACKNOLEDGEMENTS

The authors' laboratory is supported by grant PID2020-114396RB-I00 to O.P. from MICINN, Spain. L.M.-C. was recipient of a FPU fellowship from MINECO. A.R.-M. is a recipient of a Margarita Salas contract (Reference: CA1/RSUE/2021-00843) cofinanced by the Ministerio de Universidades, the "Plan de Recuperación, Transformación y Resilencia" and the Autonomous University of Madrid.

Figure legends

Figure 1. Main CLRs-induced signaling pathways. The recognition of fungal β-glucan, galectin-9, annexins or tropomyosin by dectin-1 induces phosphorylation (P) of the immunoreceptor tyrosine-based activation motif (ITAM) which facilitates the recruitment of spleen tyrosine kinase (Syk). Subsequently, activation of canonical and non-canonical nuclear factor-κB (NF-κB) results in the induction of *TNF, IL6, IL1b, IL23, IL10* and *IL2* gene expression. Dectin-1-triggering also promotes reactive oxygen species (ROS) and the specific recognition of β-glucans induce trained immunity. Dectin-2 recognizes α-mannan glycans which induce signaling pathways through ITAM-containing the adaptor molecule (e.g. Fc receptor γ‑chain (FcRγ)). The phosphorylation of ITAM recruits Syk and induces NF-κB signaling pathway modulating *TNF, IL6, IL1b* and *IL23p19* gene expression. Dectin-2-mediated activation of Syk is involved in the production of cysteinyl leukotrienes and pro-inflammatory lipid mediators. Activation of dendritic cell (DC) immunoreceptor (DCIR) by mannose and fucose-based ligands leads the phosphorylation of the immunoreceptor tyrosine-based inhibitory motifs (ITIM). The recruitment of the phosphatases SH2‑domain‑containing protein tyrosine phosphatase 1 (SHP1) or SHP2 inhibits NF-κB, STAT1-type I IFN signaling pathways, limits DCs expansion and downregulation of ITAM. Mannose receptor and DC-specific ICAM3‑grabbing non-integrin (DC-SIGN) lacks ITAM or ITIM domains. Following the recognition of mannose and fucose-based structures, N-acetylglucosamine or sulfated glycans, mannose receptor mediates phagocytosis. It is still not clear its implication in pro- or anti-inflammatory responses. DC-SIGN binds mannose and fucose inducing the inhibition of the NF-κB signaling pathway that mediates the downregulation of IL-6 and TNFα production, whereas favouring *IL10* gene expression.

Figure 2. CLRs signaling in DCs drives Th2 responses. Allergens such as Der p 1 and Der p 2 from HDM (house dust mite), Can f 1 from dog, Ara h 1 from peanut, Bla g 2 from cockroach and Fel d 1 from cat are recognized and captured by C-type lectin

receptors (CLRs) on dendritic cells (DCs) through their carbohydrates motifs and promotes the polarization towards Th2 cells. DC-SIGN: DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, TCR: T cell receptor, MHC-II: major histocompatibility complex class II.

Figure 3. Polymerized allergens conjugated to non-oxidized mannan have been developed as allergen specific-immunotherapy (AIT) vaccines. Preclinical data in human and mice showed that polymerized allergens conjugated to mannan specifically target DCs inducing the acquisition of tolerogenic and anti-allergic features, thus enhancing Treg cell polarization and Th1 responses, as well as increasing IgE-blocking antibodies. A Phase II clinical trial for the treatment of rhinitis/rhinoconjunctivitis demonstrated that a formulation against HDM allergy administered either subcutaneously or sublingually is safe and effective in achieving primary and secondary clinical outcomes. Primary outcome was the improvement of titrated nasal provocation test and secondary outcomes were the combination of symptom and medication scores and serological markers. DC: dendritic cells, PD-L1: programmed death ligand 1, DC-SIGN: DC-specific intercellular adhesion molecule 3-grabbing nonintegrin, Treg: regulatory T cell, SCIT: subcutaneous immunotherapy, SLIT: sublingual immunotherapy.

Abbreviations

AIT: Allergen-specific immunotherapy

CLRs: C-type lectin receptors

CTLD: C-type lectin-like domain

DAP12: DNAX-activation protein 12

DCIR: DC immunoreceptor

DCs: Dendritic cells

DC-SIGN: DC-specific intercellular adhesion molecule 3-grabbing non-integrin

FcR: Fc receptor

FcRγ: Fc receptor γ

FcεRI: High-affinity IgE receptor

HDM: House dust mite

HmoDCs: Human monocyte-derived DCs

IDO: Indoleamine 2,3-dioxygenase

Ig: Immunoglobulin

ILC2: Type 2 innate lymphoid cells

ITAM: Immunoreceptor tyrosine-based activating motif

ITIM: Immunoreceptor tyrosine-based inhibitory motif

MR: Mannose receptor

NF-κB: Nuclear factor-κB

PD-L1: Programmed death ligand 1

PM: Polymerized allergens conjugated to non-oxidized mannan

PRRs: Pattern-recognition receptors

ROS: Reactive oxygen species

SCIT: Subcutaneous immunotherapy,

SHP: SH2‐domain‐containing protein tyrosine phosphatase

SLIT: Sublingual immunotherapy

SOCS: Suppressor of cytokine signaling

Syk: Spleen tyrosine kinase

TLRs: Toll-like receptors

Treg: Regulatory T

References

Papers of particular interest, published recently, have been highlighted as:

•• Of major importance

1. Agache, I., et al., *Prioritizing research challenges and funding for allergy and asthma and the need for translational research-The European Strategic Forum on Allergic Diseases.* Allergy, 2019. **74**(11): p. 2064-2076.
2. Akdis, C.A., et al., *Type 2 immunity in the skin and lungs.* Allergy, 2020. **75**(7): p. 1582-1605.
3. Palomares, O., L. O'Mahony, and C.A. Akdis, *The many routes of dendritic cells to ensure immune regulation.* J Allergy Clin Immunol, 2011. **127**(6): p. 1541-2.
4. Humeniuk, P., P. Dubiela, and K. Hoffmann-Sommergruber, *Dendritic Cells and Their Role in Allergy: Uptake, Proteolytic Processing and Presentation of Allergens.* Int J Mol Sci, 2017. **18**(7).
5. Liu, E.G., et al., *Antigen-Presenting Cells in Food Tolerance and Allergy.* Front Immunol, 2020. **11**: p. 616020.
6. Schuijs, M.J., H. Hammad, and B.N. Lambrecht, *Professional and 'Amateur' Antigen-Presenting Cells In Type 2 Immunity.* Trends Immunol, 2019. **40**(1): p. 22-34.
7. Iborra, S. and D. Sancho, *Signalling versatility following self and non-self sensing by myeloid C-type lectin receptors.* Immunobiology, 2015. **220**(2): p. 175-84.
8. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
9. van Kooyk, Y. and G.A. Rabinovich, *Protein-glycan interactions in the control of innate and adaptive immune responses.* Nat Immunol, 2008. **9**(6): p. 593-601.
10. •• Keumatio Doungstop, B.C., et al., *Carbohydrates in allergy: from disease to novel immunotherapies.* Trends Immunol, 2021. **42**(7): p. 635-648.
    This revision shows the role of ligands of C-type lectin receptors and sialic acid-binding immunoglobulin-type lectins (Siglecs) in allergy.
11. Al-Ghouleh, A., et al., *The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent.* PLoS One, 2012. **7**(3): p. e33929.
12. Benito-Villalvilla, C., et al., *Novel vaccines targeting dendritic cells by coupling allergoids to mannan.* Allergo J Int, 2018. **27**(8): p. 256-262.
13. Sirvent, S., et al., *Novel vaccines targeting dendritic cells by coupling allergoids to nonoxidized mannan enhance allergen uptake and induce functional regulatory T cells through programmed death ligand 1.* J Allergy Clin Immunol, 2016. **138**(2): p. 558-567 e11.
14. •• Nieto, A., et al., *First-in-human phase 2 trial with mite allergoids coupled to mannan in subcutaneous and sublingual immunotherapy.* Allergy, 2022. **77**(10): p. 3096-3107.
    This study demonstrates for the first time that the administration of house dust mite allergoids coupled to mannan in patients is safe and successful in achieving primary and secondary clinical efficacy outcomes by both sublingual and subcutaneous routes.
15. Zelensky, A.N. and J.E. Gready, *The C-type lectin-like domain superfamily.* FEBS J, 2005. **272**(24): p. 6179-217.
16. Sancho, D. and C. Reis e Sousa, *Signaling by myeloid C-type lectin receptors in immunity and homeostasis.* Annu Rev Immunol, 2012. **30**: p. 491-529.
17. Hadebe, S., F. Brombacher, and G.D. Brown, *C-Type Lectin Receptors in Asthma.* Front Immunol, 2018. **9**: p. 733.

18. Mata-Martinez, P., M. Bergon-Gutierrez, and C. Del Fresno, *Dectin-1 Signaling Update: New Perspectives for Trained Immunity.* Front Immunol, 2022. **13**: p. 812148.
19. Kerrigan, A.M. and G.D. Brown, *C-type lectins and phagocytosis.* Immunobiology, 2009. **214**(7): p. 562-75.
20. Ahrens, S., et al., *F-actin is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells.* Immunity, 2012. **36**(4): p. 635-45.
21. Brown, G.D., J.A. Willment, and L. Whitehead, *C-type lectins in immunity and homeostasis.* Nat Rev Immunol, 2018. **18**(6): p. 374-389.
22. Bloem, K., et al., *Ligand binding and signaling of dendritic cell immunoreceptor (DCIR) is modulated by the glycosylation of the carbohydrate recognition domain.* PLoS One, 2013. **8**(6): p. e66266.
23. Richard, M., et al., *Granulocyte macrophage-colony stimulating factor reduces the affinity of SHP-2 for the ITIM of CLECSF6 in neutrophils: a new mechanism of action for SHP-2.* Mol Immunol, 2006. **43**(10): p. 1716-21.
24. Troegeler, A., et al., *C-type lectin receptor DCIR modulates immunity to tuberculosis by sustaining type I interferon signaling in dendritic cells.* Proc Natl Acad Sci U S A, 2017. **114**(4): p. E540-E549.
25. Palomares, O., et al., *Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells.* Immunol Rev, 2017. **278**(1): p. 219-236.
26. Altmann, F., *Coping with cross-reactive carbohydrate determinants in allergy diagnosis.* Allergo J Int, 2016. **25**(4): p. 98-105.
27. Peters, K. and M. Peters, *The Role of Lectin Receptors and Their Ligands in Controlling Allergic Inflammation.* Front Immunol, 2021. **12**: p. 635411.
28. Ito, T., et al., *Dectin-1 Plays an Important Role in House Dust Mite-Induced Allergic Airway Inflammation through the Activation of CD11b+ Dendritic Cells.* J Immunol, 2017. **198**(1): p. 61-70.
29. Han, W., et al., *Ovalbumin-Induced Airway Inflammation Is Ameliorated in Dectin-1-Deficient Mice, in Which Pulmonary Regulatory T Cells Are Expanded through Modification of Intestinal Commensal Bacteria.* J Immunol, 2021. **206**(9): p. 1991-2000.
30. Kanno, T., et al., *Latent 1,3-beta-D-glucan acts as an adjuvant for allergen-specific IgE production induced by Japanese cedar pollen exposure.* Allergol Int, 2021. **70**(1): p. 105-113.
31. Lilly, L.M., et al., *The beta-glucan receptor dectin-1 promotes lung immunopathology during fungal allergy via IL-22.* J Immunol, 2012. **189**(7): p. 3653-60.
32. Gour, N., et al., *Dysregulated invertebrate tropomyosin-dectin-1 interaction confers susceptibility to allergic diseases.* Sci Immunol, 2018. **3**(20).
33. Yong, H.M., et al., *Epigenetic regulation of epithelial dectin-1 through an IL-33-STAT3 axis in allergic disease.* Allergy, 2022. **77**(1): p. 207-217.
34. Gu, C., et al., *Dectin-1 Controls TSLP-Induced Th2 Response by Regulating STAT3, STAT6, and p50-RelB Activities in Dendritic Cells.* Front Immunol, 2021. **12**: p. 678036.
35. Parsons, M.W., et al., *Dectin-2 regulates the effector phase of house dust mite-elicited pulmonary inflammation independently from its role in sensitization.* J Immunol, 2014. **192**(4): p. 1361-71.
36. Barrett, N.A., et al., *Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells.* J Immunol, 2009. **182**(2): p. 1119-28.
37. Barrett, N.A., et al., *Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes.* J Exp Med, 2011. **208**(3): p. 593-604.
38. Chen, M.H., et al., *Antibody blockade of Dectin-2 suppresses house dust mite-induced Th2 cytokine production in dendritic cell- and monocyte-depleted peripheral blood mononuclear cell co-cultures from asthma patients.* J Biomed Sci, 2019. **26**(1): p. 97.

39. •• Qu, Y., et al., *Dectin-2 promotes house dust mite-skewed Th2 response through the activation of cDC2s.* Cell Immunol, 2022. **378**: p. 104558.
This study shows that house dust mite-induced Dectin-2 activation in dendritic cells promote Th2 immune responses.
40. Do, D.C., et al., *N-glycan in cockroach allergen regulates human basophil function.* Immun Inflamm Dis, 2017. **5**(4): p. 386-399.
41. Luo, X., et al., *Dendritic cell immunoreceptor drives atopic dermatitis by modulating oxidized CaMKII-involved mast cell activation.* JCI Insight, 2022. **7**(5).
42. Royer, P.J., et al., *The mannose receptor mediates the uptake of diverse native allergens by dendritic cells and determines allergen-induced T cell polarization through modulation of IDO activity.* J Immunol, 2010. **185**(3): p. 1522-31.
43. Deslee, G., et al., *Involvement of the mannose receptor in the uptake of Der p 1, a major mite allergen, by human dendritic cells.* J Allergy Clin Immunol, 2002. **110**(5): p. 763-70.
44. Cabanillas, B., et al., *Differences in the Uptake of Ara h 3 from Raw and Roasted Peanut by Monocyte-Derived Dendritic Cells.* Int Arch Allergy Immunol, 2018. **177**(1): p. 35-39.
45. Emara, M., et al., *Recognition of the major cat allergen Fel d 1 through the cysteine-rich domain of the mannose receptor determines its allergenicity.* J Biol Chem, 2011. **286**(15): p. 13033-40.
46. Kayserova, J., et al., *Selective increase in blood dendritic cell antigen-3-positive dendritic cells in bronchoalveolar lavage fluid in allergic patients.* Scand J Immunol, 2012. **75**(3): p. 305-13.
47. Zhou, Y., et al., *Mannose receptor modulates macrophage polarization and allergic inflammation through miR-511-3p.* J Allergy Clin Immunol, 2018. **141**(1): p. 350-364 e8.
48. Hsu, S.C., et al., *Functional interaction of common allergens and a C-type lectin receptor, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), on human dendritic cells.* J Biol Chem, 2010. **285**(11): p. 7903-10.
49. Zhang, Y., et al., *DC-SIGN promotes allergen uptake and activation of dendritic cells in patients with atopic dermatitis.* J Dermatol Sci, 2016. **84**(2): p. 128-136.
50. Shreffler, W.G., et al., *The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro.* J Immunol, 2006. **177**(6): p. 3677-85.
51. Kamalakannan, M., et al., *Identification and characterization of DC-SIGN-binding glycoproteins in allergenic foods.* Allergy, 2016. **71**(8): p. 1145-55.
52. Tsai, J.J., et al., *The effect of Dermatophagoides pteronyssinus group 7 allergen (Der p 7) on dendritic cells and its role in T cell polarization.* Immunobiology, 2016. **221**(11): p. 1319-28.
53. Huang, H.J., et al., *Mite allergen decreases DC-SIGN expression and modulates human dendritic cell differentiation and function in allergic asthma.* Mucosal Immunol, 2011. **4**(5): p. 519-27.
54. Emara, M., et al., *Retagging identifies dendritic cell-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN) protein as a novel receptor for a major allergen from house dust mite.* J Biol Chem, 2012. **287**(8): p. 5756-63.
55. Furmonaviciene, R., et al., *The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of in silico substrate identification and implications in allergic responses.* Clin Exp Allergy, 2007. **37**(2): p. 231-42.
56. Salazar, F., et al., *The role of lectins in allergic sensitization and allergic disease.* J Allergy Clin Immunol, 2013. **132**(1): p. 27-36.
57. Kawashima, S., et al., *beta-glucan curdlan induces IL-10-producing CD4+ T cells and inhibits allergic airway inflammation.* J Immunol, 2012. **189**(12): p. 5713-21.

58. Massoud, A.H., et al., *Dendritic cell immunoreceptor: a novel receptor for intravenous immunoglobulin mediates induction of regulatory T cells.* J Allergy Clin Immunol, 2014. **133**(3): p. 853-63 e5.
59. Peters, M., et al., *Allergy-Protective Arabinogalactan Modulates Human Dendritic Cells via C-Type Lectins and Inhibition of NF-kappaB.* J Immunol, 2016. **196**(4): p. 1626-35.
60. Spiering, R., et al., *DEC205+ Dendritic Cell-Targeted Tolerogenic Vaccination Promotes Immune Tolerance in Experimental Autoimmune Arthritis.* J Immunol, 2015. **194**(10): p. 4804-13.
61. Agache, I., et al., *Immunotherapy: State-of-the-art review of therapies and theratypes.* J Allergy Clin Immunol, 2022.
62. Durham, S.R. and M.H. Shamji, *Allergen immunotherapy: past, present and future.* Nat Rev Immunol, 2022.
63. Korotchenko, E., et al., *Laser-facilitated epicutaneous immunotherapy with hypoallergenic beta-glucan neoglycoconjugates suppresses lung inflammation and avoids local side effects in a mouse model of allergic asthma.* Allergy, 2021. **76**(1): p. 210-222.
64. Mathiesen, C.B.K., et al., *Genetically engineered cell factories produce glycoengineered vaccines that target antigen-presenting cells and reduce antigen-specific T-cell reactivity.* J Allergy Clin Immunol, 2018. **142**(6): p. 1983-1987.
65. Benede, S., et al., *Peptide Glycodendrimers as Potential Vaccines for Olive Pollen Allergy.* Mol Pharm, 2020. **17**(3): p. 827-836.
66. Palomares, F., et al., *Pru p 3-Glycodendropeptides Based on Mannoses Promote Changes in the Immunological Properties of Dendritic and T-Cells from LTP-Allergic Patients.* Mol Nutr Food Res, 2019. **63**(20): p. e1900553.
67. Rodriguez, M.J., et al., *Glycosylated nanostructures in sublingual immunotherapy induce long-lasting tolerance in LTP allergy mouse model.* Sci Rep, 2019. **9**(1): p. 4043.
68. •• Benito-Villalvilla, C., et al., *Allergoid-mannan conjugates reprogram monocytes into tolerogenic dendritic cells via epigenetic and metabolic rewiring.* J Allergy Clin Immunol, 2022. **149**(1): p. 212-222 e9.
This study shows that allergoid-mannan conjugates are able to reprogram human monocytes into tolerogenic DCs by mechanisms depending on metabolic and epigenetic rewiring, thus suggesting the potential capacity to induce trainned innate immune cells with anti-inflammatory features.
69. Benito-Villalvilla, C., et al., *Allergoid-mannan conjugates imprint tolerogenic features in human macrophages.* Allergy, 2022. **77**(1): p. 320-323.
70. Manzano, A.I., et al., *Structural studies of novel glycoconjugates from polymerized allergens (allergoids) and mannans as allergy vaccines.* Glycoconj J, 2016. **33**(1): p. 93-101.
71. Soria, I., et al., *Oral myeloid cells uptake allergoids coupled to mannan driving Th1/Treg responses upon sublingual delivery in mice.* Allergy, 2018. **73**(4): p. 875-884.
72. Benito-Villalvilla, C., et al., *Alum impairs tolerogenic properties induced by allergoid-mannan conjugates inhibiting mTOR and metabolic reprogramming in human DCs.* Allergy, 2020. **75**(3): p. 648-659.

Table 1. Main C-type lectin receptors (CLRs), localization and ligands.

Receptor	Gene name	Localization	Ligand
CLRs coupled to the spleen tyrosine kinase (Syk): HemITAM-based CLRs			
Dectin-1 (β-GR, CLECSF12)	*CLECL7A (Hu)* *Clec7a (Ms)*	DCs, monocytes, macrophages, neutrophils, $\gamma\delta$ T cells, B cells, eosinophils, mast cells, epithelium	β-1,3 glucans, galectin-9, annexins, tropomyosin
DNGR-1	*CLECL9A (Hu)* *Clec9a (Ms)*	BDCA-3$^+$ DCs (Hu), CD8α^+ DCs, tissue resident CD103 CD11b$^-$ DCs	ND
SIGNR3	*Cd209d (Ms)*	Macrophages	High mannose, fucose
CLRs coupled to the spleen tyrosine kinase (Syk): ITAM-coupled CLRs			
Dectin-2	*CLEC6A (Hu)* *Clec4n (ms)*	Macrophages, monocytes, several DC subtypes	High mannose, *a*-mannans
BDCA-2 (DLEC, CD303, CLECSF7)	*CLEC4C (Hu)*	Plasmacytoid DCs	gp120
DCAR	*Clec4b1 (Ms)*	DCs, monocytes, B cells, macrophages	ND
Mincle	*CLEC4E (Hu)* *Clec4e (Ms)*	Macrophages, neutrophils	*a*-mannose, glycolipids, SAP-130
MDL-1 (CLECSF5)	*CLEC5A (Hu)* *Clec5a (Ms)*	Monocytes, macrophages, osteoclasts	ND
CLRs containing ITIM			
DCIR (CLECS-F6, LLIR) Dcir1 (DCIR, Clecf6) Dcir2 (33D1)	*CLEC4A (Hu)* *Clec4a2 (Ms)* *Clec4a4 (Ms)*	Monocytes, , B cells, macrophages, DCs, granulocytes	Mannose, fucose
MICL (DCAL-2, KLRL1, CLL1)	*CLECL12A (Hu)* *Clec12a (Ms)*	Granulocytes, DCs, monocytes, macrophages	ND
Macrophage antigen H (CLECL12B)	*CLECL12B (Hu)* *Clecl12b (Ms)*	Macrophages	Surface ligands upregulated following DNA damage
CLRs without ITAM or ITIM			
Mannose receptor (MR, MMR, CD206)	*MRC1 (Hu)* *Mrc1 (Ms)*	Macrophages, monocyte-derived DCs, smooth muscle cells, epitelial cells, mesangial cells	High mannose, fucose, sLex, N-acetyl glucosamine, dulfated glycans
DC-SIGN (CLEC4L)	*CD209 (Hu)*	DCs, macrophages	High mannose, fucose
DEC-205 (CD205)	*LY75 (Hu)* *Ly75 (Ms)*	CD8a$^+$ DCs, B cells, macrophages, T cells, granulocytes	PLA (*Y. pestis*), K12 (*E. coli*)
SIGNR1	*Cd209b (Ms)*	Macrophages, lamina propria DCs	Dextran, mannan, fucose

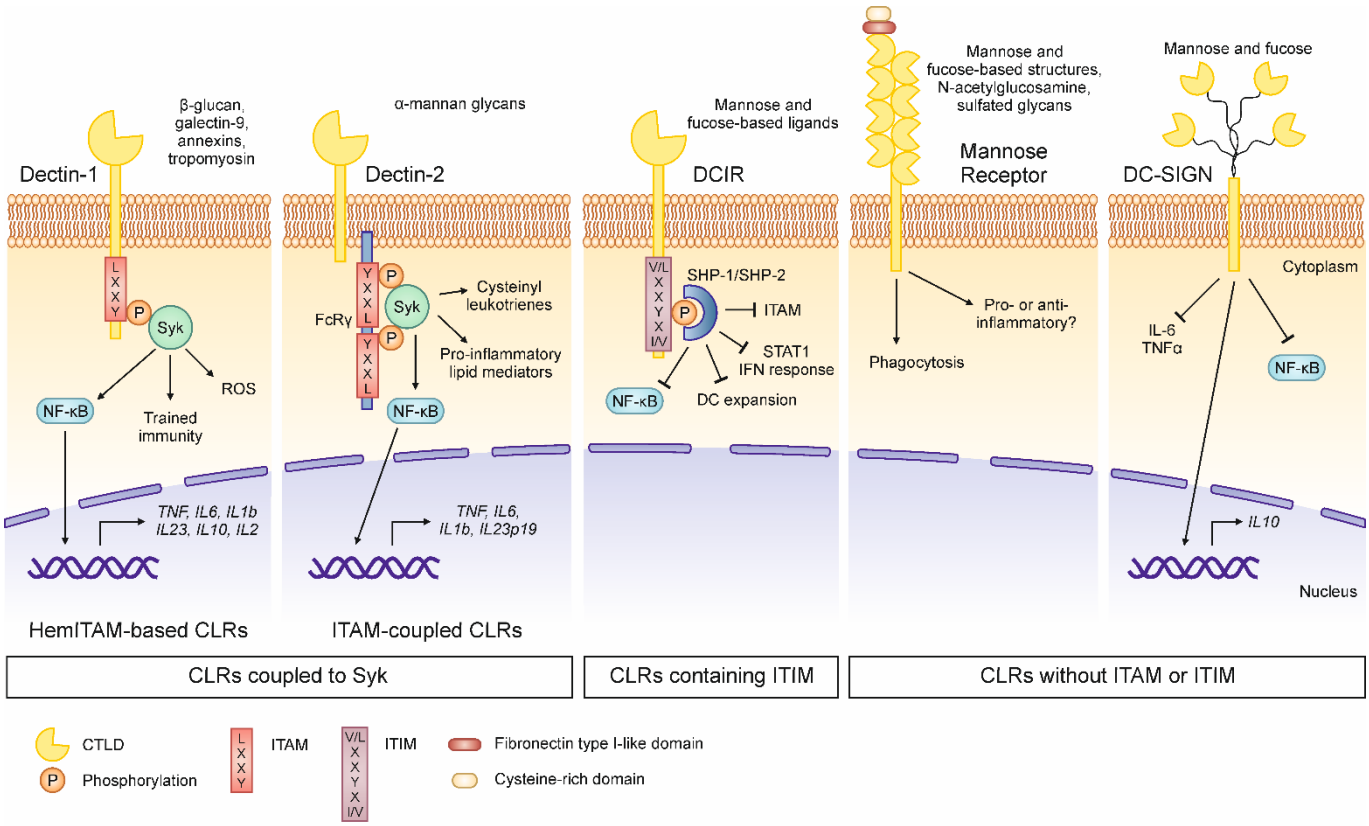
β-glucan, galectin-9, annexins, tropomyosin
Dectin-1
α-mannan glycans
Dectin-2
Mannose and fucose-based ligands
DCIR
Mannose and fucose-based structures, N-acetylglucosamine, sulfated glycans
Mannose Receptor
Mannose and fucose
DC-SIGN
Cytoplasm
Nucleus
Syk
NF-κB
ROS
Trained immunity
TNF, IL6, IL1b IL23, IL10, IL2
FcRγ
Cysteinyl leukotrienes
Pro-inflammatory lipid mediators
TNF, IL6, IL1b, IL23p19
SHP-1/SHP-2
ITAM
STAT1
IFN response
DC expansion
Phagocytosis
Pro- or anti-inflammatory?
IL-6
TNFα
IL10
HemITAM-based CLRs
ITAM-coupled CLRs
CLRs coupled to Syk
CLRs containing ITIM
CLRs without ITAM or ITIM
CTLD
Phosphorylation
ITAM
ITIM
Fibronectin type I-like domain
Cysteine-rich domain

Figure 1

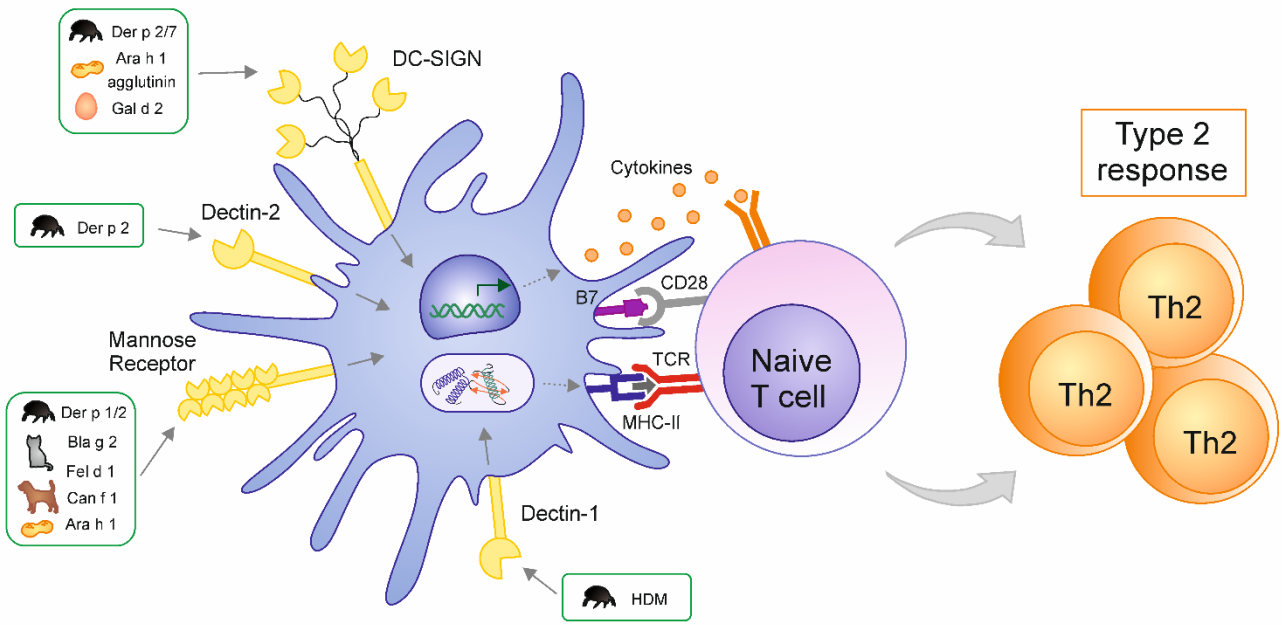
Der p 2/7
Ara h 1
agglutinin
Gal d 2
DC-SIGN
Der p 2
Dectin-2
Mannose
Receptor
Der p 1/2
Bla g 2
Fel d 1
Can f 1
Ara h 1
Cytokines
B7
CD28
TCR
MHC-II
Naive
T cell
Dectin-1
HDM
Type 2
response
Th2
Th2
Th2

Figure 2

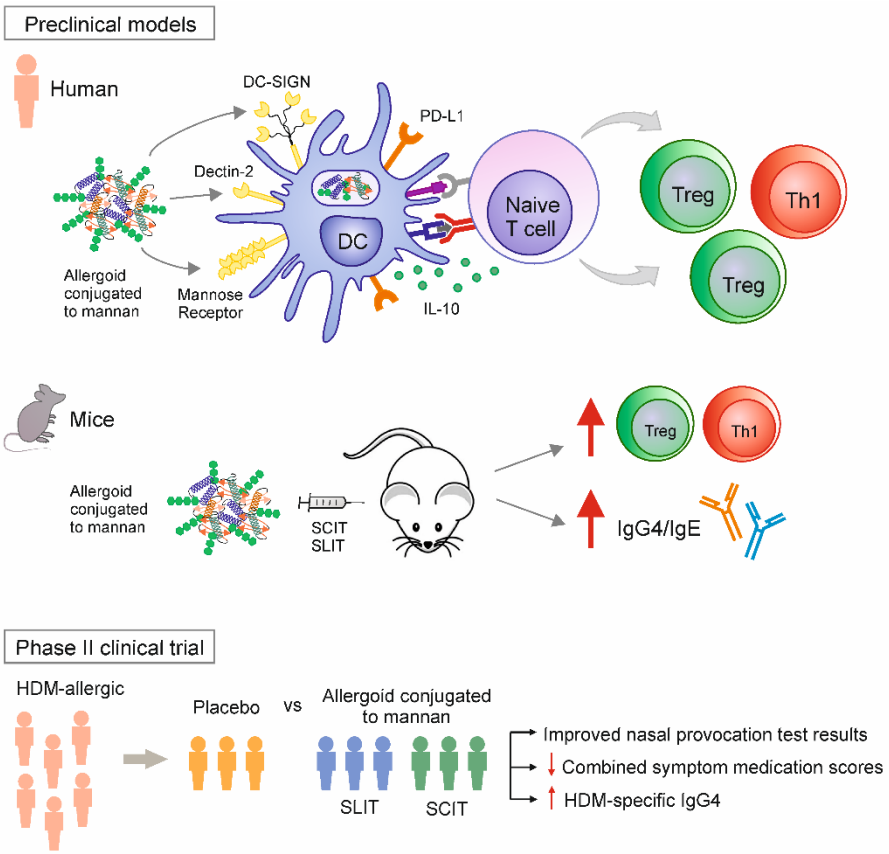
Preclinical models
Human
DC-SIGN
PD-L1
Dectin-2
DC
Naive
T cell
Treg
Th1
Treg
Allergoid
conjugated
to mannan
Mannose
Receptor
IL-10
Mice
Allergoid
conjugated
to mannan
SCIT
SLIT
Treg
Th1
IgG4/IgE
Phase II clinical trial
HDM-allergic
Placebo
vs
Allergoid conjugated
to mannan
SLIT
SCIT
Improved nasal provocation test results
Combined symptom medication scores
HDM-specific IgG4

Figure 3