**UNIVERSIDAD COMPLUTENSE DE MADRID**
FACULTAD DE CIENCIAS QUÍMICAS


# TESIS DOCTORAL

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## Molecular mechanisms involved in the mode of action of allergoid-mannan conjugates and anti-IgE treatments in human dendritic cells

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid

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A mis padres y hermana

A Alberto

# AGRADECIMIENTOS

*"Después de escalar una montaña muy alta, descubrimos que hay muchas más montañas por escalar"* Nelson Mandela

Se podría decir que esto es lo que siento ahora mismo, con estas palabras termino de escalar una montaña muy alta. Pero tras este gran esfuerzo, soy consciente de que aún quedan muchas batallas por luchar, y muchas montañas por escalar. Sin duda, para escalar esta, la montaña más alta hasta la fecha, he contado con la ayuda de numerosas personas a las que quiero agradecer.

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# TABLE OF CONTENTS

# ABBREVIATIONS

**1-MT:** 1-methyl tryptophan

**AIT**: Allergen-specific Immunotherapy

**Alum**: Aluminium hydroxide

**APC**: Antigen-presenting cell

**AR**: Allergic rhinitis

**ARIA**: Allergic Rhinitis and its Impact in Asthma

**Breg**: Regulatory B

**BSA**: Bovine serum albumin

**CCR7**: C-C chemokine receptor 7

**cDC**: Conventional dendritic cell

**CFSE**: Carboxifluorescein diacetate N-succinimidyl ester

**ChIP**: Chromatin immunoprecipitation

**CL**: IgE-FcεRI-crosslinker

**CLR**: C-type lectin receptor

**CRSwNP**: Chronic rhinosinusitis with polyposis

**CSU**: Chronic spontaneous urticaria

**CTLA-4**: Cytotoxic T-lymphocyte antigen 4

**DAMP**: Damage-associated molecular pattern

**DARPin**: Designed ankyrin repeat protein

**DC**: Dendritic cell

**DC-SIGN**: Specific intercellular adhesion molecule-3-grabbing non-integrin

**DNMTs**: DNA methyltransferases

**EF1α**: Elongation factor 1 α

**FcεR**: Fcε receptor

**FLT3L**: FMS-like tyrosine kinase ligand 3

**FOXP3**: Forkhead box P3

**GINA**: Global Initiative for Asthma

**GM-CSF**: Granulocyte monocyte-colony stimulating factor

**H3K27ac**: Histone 3 lysine 27 acetylation

**H3K27me3**: Histone 3 lysine 27 trimethylation

**H3K4me3**: Histone 3 lysine 4 trimethylation

**HATs**: Histone acetyltransferases

**HDACs**: Histone deacetylases

**HmoDC**: Human monocyte-derived dendritic cell

**HMTs**: Histone methyltransferases

**HR2**: Histamine receptor 2

**IC**: Immune complexes

**IDO**: Indoleamine 2,3-dioxygenase

**Ig**: Immunoglobulin

**IL**: Interleukin

**ILC**: Innate lymphoid cell

**IRF3**: Interferon regulatory transcription factor 3

**$K_D$**: Dissociation constant

**Kyn**: Kynurenine

**LAG3**: Lymphocyte activation gene 3

**Lige**: Ligelizumab

**LPS**: Lipopolysaccharide

**mAb**: Monoclonal antibody

**Mannan-tolDC**: DCs differentiated in the presence of allergoid-mannan conjugates

**MCT**: Microcrystalline tyrosine

**mDC**: Myeloid dendritic cell

**MFI**: Mean fluorescence intensity

**MHC**: Major histocompatibility complex

**MiRNA**: microRNA

**MØ**: Macrophage

**MPL**: Monophosphoryl lipid A

**mTOR**: Mammalian target of rapamycin

**NIP**: 4-hydroxy-3-iodo-5-nitrophenylacetyl

**NK**: Natural killer

**NKT**: Natural killer T cell

**NLRP3**: NOD-like receptor family pyrin domain containing 3

**NRP1**: Neuropilin 1

**OCR**: Oxygen consumption rate

**ODN**: Oligodeoxynucleotide

**Oma**: Omalizumab

**OXPHOS**: Oxidative phosphorylation

**PAMP**: Pathogen-associated molecular pattern

**PBLs**: Peripheral blood lymphocytes

**PBMC**: Peripheral blood mononuclear cells

**pDC**: Plasmacytoid dendritic cell

**PD1**: Programmed death 1

**PDCD4**: Programmed cell death protein 4

**PD-L1**: Programmed death ligand 1

**PI3K**: Phosphoinositide 3-kinase

**PM**: Allergoid-mannan conjugates

**PMA**: Phorbol myristate acetate

**PPP**: Pentose phosphate pathway

**PRR**: Pattern-recognition receptor

**pTreg**: Peripheral-derived regulatory T cell

**RAST**: Allergen-specific IgE antibody test

**ROS**: Reactive oxygen species

**SAMP**: Self-associated molecular pattern

**SOCS**: Suppressor of cytokine signalling

**TCA**: Tricarboxylic acid

**Tfh**: T follicular helper

**TGF-β**: Transforming growth factor β

**Th**: T helper

**TIGIT**: T cell immunoreceptor with immunoglobulin and ITIM domains

**TIM3**: T cell immunoglobulin and mucin domain-containing 3

**TLR**: Toll-like receptor

**TLR9-L**: Toll-like receptor 9-ligand

**TNF**: Tumor necrosis factor

**Tol-DCs**: Tolerogenic DCs

**Treg**: Regulatory T

**TSDR**: Treg-specific demethylation region

**TSLP**: Thymic stromal lymphopoietin

**tTreg**: Thymus-derived regulatory T cell

**VLP**: Viral-like particle

**WHO**: World health organization

# RESUMEN/ABSTRACT

# RESUMEN

La alergia se define como una reacción exagerada a sustancias externas (alérgenos) mediada por el sistema inmune. Las enfermedades alérgicas constituyen un problema muy importante de salud con una gran carga socioeconómica, disminuyendo la calidad de vida de los pacientes. Actualmente, la inmunoterapia específica de alérgeno (AIT) es el único tratamiento con capacidad potencial para modificar el curso de las enfermedades alérgicas a largo plazo, y junto a los biológicos como los anticuerpos monoclonales (mAbs) dirigidos frente a dianas específicas, representan las terapias más efectivas para los pacientes en los que la medicación convencional no es suficiente para el control de los síntomas. Sin embargo, estos tratamientos aún presentan ciertas limitaciones en cuanto a eficacia, seguridad, larga duración o alto coste, por lo que hay una necesidad urgente de desarrollar tratamientos más efectivos y seguros. Los alergoides conjugados a manano son vacunas de nueva generación para AIT dirigidas frente a células dendríticas (DCs) capaces de inducir células T reguladoras (Treg) funcionales. Por otro lado, omalizumab, un biológico anti-IgE, está aprobado para el tratamiento del asma alérgica grave, de urticaria crónica espontánea (CSU) y de rinosinusitis crónica con pólipos nasales (CRSwNP). Recientemente, se ha desarrollado un nuevo mAb anti-IgE de alta afinidad (ligelizumab) que se encuentra actualmente en fases muy avanzadas para el tratamiento de CSU.

Las DCs son células presentadoras de antígeno profesionales que tienen un papel clave en la inducción de respuestas inmunes conectando el sistema inmune innato y el adaptativo. En alergia, las DCs contribuyen tanto a la patofisiología de la enfermedad como a la inducción de tolerancia frente a alérgenos. El mantenimiento de la tolerancia tras el tratamiento con AIT requiere la generación de células Treg y células B reguladoras (Breg), así como de anticuerpos bloqueantes específicos de alérgeno. Estudios recientes han demostrado que omalizumab induce un aumento de las células Treg en niños con asma grave que correlaciona con el control de su enfermedad. Sin embargo, los mecanismos subyacentes se desconocían. Teniendo en cuenta todos estos aspectos, el objetivo global de esta Tesis Doctoral es dilucidar los mecanismos moleculares mediante los cuales nuevas vacunas para AIT (alergoides conjugados a manano) y biológicos anti-IgE (omalizumab y ligelizumab) pueden influenciar la función de las DCs y su capacidad para generar células Treg funcionales y así mejorar nuestro conocimiento acerca de los mecanismos implicados en la generación de tolerancia a alérgenos.

En este trabajo hemos demostrado que el aluminio, el adyuvante más usado en AIT, inhibe las propiedades tolerogénicas inducidas por alergoides conjugados a manano (PM) en DCs humanas mediante la inhibición de la activación de mTOR, la reprogramación metabólica de las células y la inhibición de la producción de especies reactivas de oxígeno (ROS) en DCs activadas con PM, disminuyendo así su capacidad para inducir células Treg funcionales. Además, hemos demostrado que la diferenciación de monocitos de donantes no atópicos o alérgicos hacia DCs en presencia de PM da lugar a DCs tolerogénicas estables (mannan-tolDCs) que inducen un mayor número de células Treg en comparación con DCs convencionales. Dichas mannan-tolDCs presentan un cambio en el metabolismo de glucosa desde un efecto Warburg con producción de lactato hacia fosforilación oxidativa mitocondrial y un cambio en las marcas epigenéticas en comparación con DCs diferenciadas en ausencia de la vacuna de AIT. Del mismo modo, los alergoides conjugados a manano inducen la generación de macrófagos (MØ) humanos productores de IL-10 con fenotipos reguladores, capaces de inducir y expandir células Treg específicas de alérgeno.

Por otro lado, hemos demostrado que omalizumab, un mAb anti-IgE, retira la IgE unida a la membrana de DCs plasmacitoides (pDCs), reduciendo la producción de TNF-α y restaurando la expresión de IDO, IFN-α, y su capacidad para generar células Treg tras el entrecruzamiento del receptor de alta afinidad FcεRI mediado por IgE y la activación con ligando de TLR9 (TLR9-L). Hemos observado que ligelizumab, un nuevo mAb anti-IgE en desarrollo, también restaura la capacidad de pDCs activadas con TLR9-L para inducir la generación de células Treg tras el entrecruzamiento IgE-FcεRI, y no hemos detectado diferencias significativas entre los efectos de ligelizumab y omalizumab en dicha restauración de la generación de células Treg tras el entrecruzamiento IgE-FcεRI.

En resumen, en esta Tesis Doctoral descubrimos nuevos mecanismos moleculares mediante los cuales la AIT y los mAbs anti-IgE inmunomodulan DCs humanas y su capacidad de generar células Treg funcionales. Hemos demostrado que el aluminio inhibe las propiedades tolerogénicas inducidas por PM. Hemos mostrado por primera vez que alergoides conjugados a manano alteran la diferenciación de monocitos de individuos no atópicos o alérgicos hacia DCs y MØ tolerogénicos estables. Además, hemos dilucidado un nuevo mecanismo molecular que puede ayudar a explicar cómo el tratamiento con omalizumab induce un aumento de células Treg en niños asmáticos, y demostrado que ligelizumab también tiene capacidad de alterar la inducción de células Treg. Los datos presentados en esta Tesis Doctoral muestran nuevos mecanismos mediante los cuales alergoides conjugados a manano inducen tolerancia a alérgenos y

podrían contribuir a mejorar los tratamientos actuales de AIT. Además, nuestros datos acerca del mecanismo de mAbs anti-IgE contribuyen a mejorar el conocimiento del modo de acción de omalizumab y podrían ayudar a identificar nuevos biomarcadores potenciales para monitorizar los tratamientos anti-IgE.

# ABSTRACT

Allergy is defined as the unexpected abnormal or exaggerated reaction to an exogenous stimulus (allergen) involving the immune system. Allergic diseases represent a major health problem of increasing prevalence with high socio-economic burden, decreasing the quality of life of many patients. Nowadays, allergen-specific immunotherapy (AIT) is the only treatment with potential long-lasting disease-modifying effects for allergic diseases, and together with biologicals such as monoclonal antibodies (mAbs), represent the most effective therapies for allergic patients when conventional medication is not enough to control the symptoms. However, they still have drawbacks in terms of efficacy, security, long duration or high cost. Therefore, there is an urgent need to develop more effective and safer treatments. Allergoid-mannan conjugates represent next-generation vaccines for AIT targeting dendritic cells (DCs) able to promote the generation of functional regulatory T (Treg) cells. On the other hand, omalizumab, an anti-IgE biological, is approved for the treatment of severe allergic asthma, for chronic spontaneous urticaria (CSU) and for chronic rhinosinusitis with nasal polyps (CRSwNP). Recently, a next-generation anti-IgE mAb (ligelizumab) with significant higher affinity for IgE than omalizumab, has been developed and it is being currently assayed in phase III random clinical trials for CSU.

DCs are professional antigen-presenting cells with a key role in the orchestration of proper immune responses by linking innate and adaptive immunity. In allergy, DCs display the dual capacity to contribute both to the pathophysiology and to the induction of allergen tolerance. Remarkably, sustained tolerance after AIT requires the generation of Treg and regulatory B (Breg) cells as well as allergen-specific blocking antibodies. Recent findings demonstrated that omalizumab induces an increase in Treg cells in asthmatic children, which correlates with asthma control. However, the underlying mechanisms remained unknown. Considering all these aspects, the global aim of this Doctoral Thesis is to study the molecular mechanisms by which next generation AIT vaccines (allergoid-mannan conjugates) and anti-IgE biologicals (omalizumab and ligelizumab) might influence DC's function and their capacity to generate functional Treg cells to provide novel insights into the mechanisms involved in the generation of allergen tolerance.

In this work we have demonstrated that alum, the most widely used adjuvant in AIT, impairs the tolerogenic properties induced by allergoid-mannan conjugates (PM) in human DCs. Mechanistically, we showed that alum inhibits mTOR activation, alters the

metabolic reprogramming of cells by shifting glycolytic pathways, and inhibits reactive oxygen species (ROS) production in PM-activated DCs, thus impairing their capacity to generate functional Treg cells. We have also shown that the differentiation of monocytes from nonatopic and allergic subjects into DCs in the presence of PM yields stable tolerogenic DCs (mannan-tolDCs) that induce higher numbers of Treg cells than conventional DCs. In addition, mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation and display epigenetic reprogramming compared to DCs differentiated in the absence of the AIT vaccine. Likewise, allergoid-mannan conjugates promote the generation of human IL-10-producing macrophages (MØ) with regulatory phenotypes that are able to induce and expand allergen-specific Treg cells.

We have also demonstrated that omalizumab, an anti-IgE mAb, removes membrane-bound IgE on plasmacytoid DCs (pDCs), thus downregulating TNF-α production and restoring the expression of IDO, IFN-α, and their capacity to generate Treg cells under IgE-mediated FcεRI-crosslinking and TLR9-ligand (TLR9-L) stimulation. We have observed that ligelizumab, a novel anti-IgE mAb, also displays the capacity to restore the ability of TLR9-L-activated pDCs to generate Treg cells after IgE-FcεRI crosslinking. We have not detected significant differences between the effects of ligelizumab and omalizumab in the restoration of Treg cells after IgE-FcεRI crosslinking.

In summary, in this Doctoral Thesis we have uncovered previously unknown molecular mechanisms by which AIT and anti-IgE mAbs immunomodulate human DCs and their capacity to generate functional Treg cells. We have demonstrated that alum impairs the tolerogenic properties induced by allergoid-mannan conjugates. We have shown for the first time that allergoid-mannan conjugates reprogram nonatopic and allergic monocyte differentiation into stable tolerogenic DCs and MØ. In addition, we have provided a molecular mechanism that might well help to explain how omalizumab treatment increases Treg cells frequency in asthmatic children, and demonstrated the capacity of ligelizumab to also affect Treg cell generation. The data presented in this Doctoral Thesis shed light into novel mechanisms by which allergoid-mannan conjugates induce allergen tolerance, which might well contribute to improve the formulation of AIT vaccines. In addition, our findings regarding the mechanisms of anti-IgE mAbs enhance our mechanistic knowledge of the mode of action of anti-IgE mAbs and might well help to discover potential novel biomarkers to assess the clinical efficacy of anti-IgE treatments.

# INTRODUCTION

# 1. Allergy

The term “Allergy” was coined by the Austrian paediatrician Clemens von Pirquet on July 24, 1906 as “specifically altered reactivity of the organism” induced by a foreign substance that he termed as “allergen”.[1] Today, allergy is defined as the unexpected abnormal or exaggerated reaction to an exogenous stimulus involving immune system.[2] Allergy is a type of hypersensitivity reaction that can display immune memory-driven mechanisms or immune-driven non memory mechanisms. Among the immune memory-driven mechanisms, there are IgE-mediated (type I hypersensitivity) or non-IgE mediated allergies (type II/III/IV hypersensitivity).[3] Allergy is not a disease itself, but a mechanism leading to disease in response to non-microbial environmental antigens, also called allergens, which are innocuous to non-allergic people. In clinical practice, allergy can manifest as anaphylaxis, urticaria, angioedema, allergic rhinoconjunctivitis, allergic asthma, serum sickness, allergic vasculitis, hypersensitivity pneumonitis, atopic dermatitis (eczema), contact dermatitis and granulomatous reactions, as well as the wide spectrum of food- or drug–induced hypersensitivity reactions.[4, 5]

Allergy is a major health problem of increasing prevalence worldwide, mainly due to environmental factors, behavioral changes, or life-style.[6] In Europe, allergy is the most common chronic disease affecting 100 million people with allergic rhinitis, 7 million people with food allergy, and 70 million people with asthma.[7] The propensity to develop allergies is influenced by the inheritance of several genes, being the full inheritance pattern multigenic. However, it is clear that environmental influences have a significant impact on the development of allergy, and they synergize with genetic risk factors. For instance, exposure to microbes during early childhood may reduce the risk for developing allergies (hygiene hypothesis) and respiratory viral and bacterial infections are a predisposing factor in the development of asthma or exacerbations of pre-existing asthma.[6, 8] Allergy affects the quality of life of patients, especially when they suffer from the most severe phenotypes. Failure to diagnose asthma and allergic diseases leads to uncontrolled diseases and, consequently, higher sanitary costs.[7, 9] Therefore, there is an urgent need to develop better therapies as allergen-specific immunotherapy (AIT) or biological treatments.[9, 10]

Although allergy has been considered a disease mediated mainly by T helper (Th) 2 lymphocytes, recent studies demonstrate that allergic diseases are complex both in physiopathology and etiology.[11, 12] Therefore, allergy displays different phenotypes and endotypes.[12, 13] A phenotype is defined as the observable properties of a patient that are

produced by the interaction of the genotype with the environment, whereas endotypes are subtypes of a condition, defined by a distinct functional or pathophysiological mechanism.[13, 14] The knowledge of the immunological mechanisms underlying allergic diseases and potential curative treatment alternatives has experienced exciting advances over the last decades.[10]

## 1.1. The underlying mechanisms in allergy

Allergens interact with various parts of the innate immune system, which plays a fundamental role in shaping adaptive immune responses. The immunogenic basis of allergic diseases involves two different and consecutive stages: the sensitization phase and the re-exposure phase. The re-exposure phase comprises two types of events: early acute and late-phase reactions. Perpetuation of the inflammation of affected tissues along the re-exposure phase is associated with chronicity, tissue remodeling, and the more severe clinical manifestations.[4, 15, 16]

i) The *sensitization phase* is induced upon the first exposure to an allergen and it is characterized by the absence of clinical symptoms (**Figure I.1**). In genetically predisposed patients, dendritic cells (DCs) located in the respiratory and gastrointestinal tracts and skin, capture the allergens and process them while migrating to the closer lymph nodes, where they induce the generation of allergen-specific effector Th2 cells. These Th2 cells produce interleukin (IL)-4 and IL-13 that induce class switching in B cells to produce specific immunoglobulin (Ig)E antibodies that bind to the high affinity Fcε receptor I (FcεRI) on mast cells and basophils, thus leading to the patient's sensitization. Besides, allergen-specific memory Th2 and B cells are generated.[15, 16] Recent studies have demonstrated that disruption of tight junctions and the consequent alteration in the epithelial barrier integrity favour the accumulation of allergens and other environmental factors in the submucosa, boosting the previous

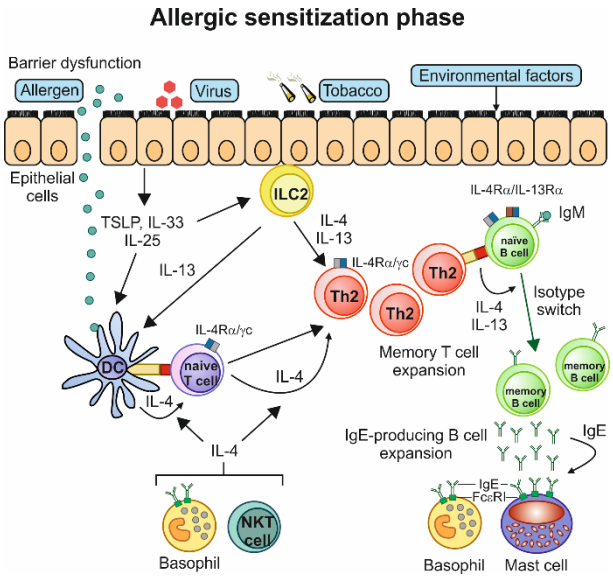


**Figure I.1. Pathophysiology of allergic sensitization phase**. During the sensitization phase, allergens are uptaken by DCs and induce a series of events leading to IgE production by B cells and memory Th2 cells expansion. *DC* Dendritic cell, *Th* T helper cell, *ILC2* Type 2 innate lymphoid cell, *NKT* Natural killer T cell, *TSLP* Thymic stromal lymphopoietin.

effects.[17, 18] Besides, epithelial cells produce cytokines known as alarmins (IL-25, IL-33, and thymic stromal lymphopoietin [TSLP]) that stimulate DCs promoting Th2 responses and activating type 2 innate lymphoid cells (ILC2s) (**Figure I.1**).[19]

ii) The *re-exposure phase* takes place when there is a second encounter with the causative allergen, which induces a crosslinking of IgE-FcεRI complexes on sensitized basophils and mast cells, generating their activation, degranulation, and the rapid release of different mediators such as histamine, proteases, proteoglycans, leukotrienes, etc. These mediators collectively induce increased vascular permeability and inflammation, vasodilation, bronchial and visceral smooth muscle contraction, and mucus production. This reaction is initiated within the first minutes upon allergen exposure and it is known as the *immediate hypersensitivity reaction* (**Figure I.2**). Immediate hypersensitivity reactions are manifested in different ways, depending on the tissues affected, including rashes, sinus congestion, bronchial constriction, abdominal pain, diarrhoea, and systemic shock. In the most extreme systemic form, called anaphylaxis, mast cell–derived mediators can restrict airways to the point of asphyxiation and produce cardiovascular collapse leading to death.[15] After the hypersensitivity reaction, in some patients, *late-phase responses* might take place (**Figure I.2**). It is a more slowly developing inflammatory component characterized by the accumulation of eosinophils, neutrophils, macrophages (MØ), and Th2 cells. This phase is mainly triggered by the release of mediators and the activation of allergen-specific memory Th2 cells, via the IgE-facilitated allergen presentation by antigen presenting cells (APCs) such as DCs and B cells.[15, 20, 21] Alarmins produced by epithelial cells activate ILC2s to produce IL-5 and IL-13, contributing to the described effects. All these mechanisms explain the phenotypes/endotypes mediated by type 2 immunity.[4] However, recent studies have demonstrated that other populations as IFN-γ-producing Th1 lymphocytes and IL-17-producting Th17 lymphocytes, together with natural killer T cells (NKT), favours the apoptosis of epithelial cells and neutrophils infiltration to the tissues, contributing to the clinical manifestations of allergic patients with phenotypes/endotypes mediated by non-type 2 immunity (**Figure I.3**).[16, 22, 23] Repeated

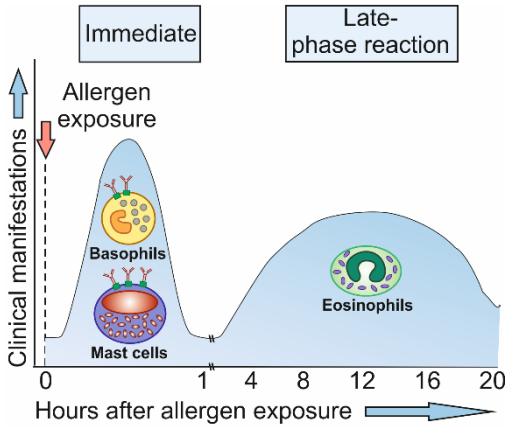


**Figure I.2. Kinetics of the immediate and late-phase reactions of allergy.** The immediate reaction occurs within minutes after challenge (allergen exposure in a previously sensitized individual), and the late-phase reaction develops 2 to 24 hours later. Adapted from *Cellular and Molecular Immunology (A.K.Abbas).*

bouts of these reactions can lead to chronic allergic diseases, with tissue damage and remodelling.[24, 25]

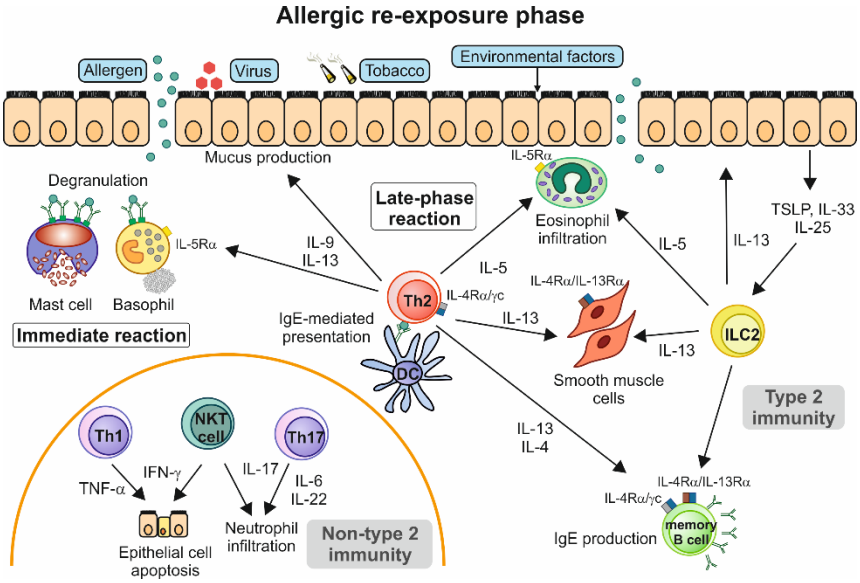


**Figure I.3. Pathophysiology of allergic re-exposure phase**. In individuals who are sensitized to the allergen, subsequent allergen exposure induces an immediate reaction of mediators released by mast cells and basophils, leading to the acute symptoms. Afterwards, cytokine secretion by memory Th2 cells within a few hours leads to more symptoms. *DC* Dendritic cell, *Th* T helper cell, *ILC2* Type 2 innate lymphoid cell, *NKT* Natural killer T cell, *TSLP* Thymic stromal lymphopoietin.

### *1.1.1. The role of IgE*

In 1919, Ramirez reported that a patient who had received a blood transfusion from a horse allergic donor, became allergic to horses.[26] In 1921, Prausnitz and Küstner demonstrated passive sensitization of the skin, since them referred as PK-test and identified a serum factor involved, which they called reagin.[27] For 45 years, there was an unsuccessful search of the factor in plasma that caused the positive PK-test. In the 1960's, K. and T. Ishizaka described a new Ig isotype in serum from allergic patients which they named gamma E.[28] In 1965, S.G.O. Johansson detected in a serum from a myeloma patient, an M-component different from the already described four immunoglobulins,[29] and it was in 1968 when a meeting in the World Health Organization (WHO) International Reference Centre resulted in the publication of the official identification of the fifth immunoglobulin, IgE.[30, 31] IgE mediates type I hypersensitivity reactions and it is the immunoglobulin isotype that has the lowest abundance *in vivo.*[32] IgE levels are tightly regulated to avoid the induction of undesired anaphylaxis, with a half-life in serum of 2.5 days.[33] IgE signals through two different FcεRs, the high-affinity receptor FcεRI and the low-affinity receptor FcεRII, also known as CD23.[34] FcεRI is expressed by mast cells and basophils as αβγ2 tetramer, on which its activation induces degranulation, eicosanoid production and cytokine production. In humans, this receptor

is also expressed by DCs, MØ, and eosinophils as a trimeric αγ2 form, where its activation mediates the internalization of IgE-bound allergens for processing and preservation on the cell surface, as well as the production of type 2 cytokines. On the other side, CD23 is expressed mainly by B cells but also on other cells such as MØ and epithelial cells.[35-37]

## 1.2. Allergic diseases

Allergic diseases represent a collection of disorders mediated by the innate and adaptive immune responses previously described. Major of allergic diseases are mediated by type 2 inflammation.[38] The main manifestations of allergic diseases are allergic rhinitis, asthma, atopic dermatitis, and food allergies.[9]

### *1.2.1. Allergic rhinitis*

Allergic rhinitis (AR) is a prevalent health problem in both adults and paediatric patient populations. It is perhaps the most common allergic disease affecting to 20-30% of adults and up to 40% of children, and reducing their quality of life.[4] Apart from upper airway symptoms, it implies sleeping and psychological disturbances, decreased work productivity and school performance. AR is a well-defined endotype according to ARIA (Allergic Rhinitis and its Impact in Asthma) guidelines.[39] It is defined as an IgE-mediated, type 1 hypersensitivity response to common allergens such as plant pollen or house dust mites localized in the upper respiratory tract by inhalation.[40] It is characterized by anterior and posterior rhinorrhoea, nasal congestion, itching of the nose, and sneezing occurring for more than one hour or two or more consecutive days. The most common comorbidities associated with AR are asthma and conjunctivitis.[4] The clinical expression of the disease can be explained by a cascade of immunological and biochemical events. Allergens are inhaled and diffuse into nasal tissues, where they are captured by DCs.[4, 40, 41]

Currently, the most common diagnostic tools for AR are percutaneous skin test (skin prick test) and the allergen-specific IgE antibody test (RAST). Medical therapy includes allergen avoidance, symptomatic relief, anti-inflammatory therapies and AIT. Intranasal corticosteroids are used both in adults and children, which are safe and more effective than the combination of oral antihistamines and leukotriene receptor antagonists. Combination of inhaled corticosteroids and intranasal antihistamines has been shown to be even more effective than each treatment alone.[39] AIT is a standard therapeutic strategy for AR patients whose symptoms persist despite the use of

conventional medication. The clinical efficacy of AIT has been well established through numerous double-blind, placebo-controlled clinical trials. Both subcutaneous and sublingual immunotherapy induce a reduction in the symptoms and medication use, especially in children with moderate-to-severe AR despite appropriate pharmacotherapy.[4, 41, 42] Omalizumab, an anti-IgE humanized monoclonal antibody (mAb), which is approved for severe allergic asthma, chronic spontaneous urticaria (CSU) and chronic rhinosinusitis with nasal polyps (CRSwNP), is able to reduce nasal and ocular symptoms in AR but has not been approved for the treatment of AR yet.[43]

### *1.2.2. Allergic asthma*

Allergic asthma is a heterogeneous chronic inflammatory disease of the airways characterized by airflow obstruction (normally reversible) accompanied by airway hyperresponsiveness and local inflammation.[44] The main symptoms encompass recurrent episodes of wheezing, coughing, chest tightness, as well as acute exacerbations associated with breathlessness. The prevalence of asthma has dramatically increased over the last decades, affecting almost 400 million people worldwide whose quality of life is significantly impaired.[45-47] Regarding the exposure trigger, asthma can be allergic or non-allergic depending on whether or not there are clinical allergic reaction and *in vitro* IgE response to specific aeroallergens. Allergic asthma is a consequence of gene-environment interactions and bursts are frequently triggered by allergen exposure. Severe asthma is an uncontrolled asthma despite adherence with maximal optimized high dose of inhaled corticosteroids and long-acting β2-agonist treatment and management of contributory factors. It is a subset of difficult-to-treat asthma.[44, 48] An asthma exacerbation is considered to be an increase in the patient's asthma symptoms with increasingly impaired lung function that require augmented medication, a visit to the physician, or hospitalization. Recurrent asthma exacerbations lead to a progressive loss of lung function. Viral infections are common triggers of exacerbations in asthma and may impair asthma symptom control. In addition to viral infections, bacterial infections on the respiratory tract have been associated with worsening of symptoms in asthma patients.[49] The absenteeism and the high healthcare resource needed to manage the disease make the economic burden of asthma one of the highest among all chronic diseases.[44, 47]

For the resolution of the acute symptoms of the disease, the recommendation is avoidance of the allergen in the environment. Besides, clinical therapy includes anti-inflammatory and bronchodilator treatments. In particular, inhaled corticosteroids alone or in combination with long-acting bronchodilators. GINA (Global Initiative for Asthma)

2021 guidelines recommend that all asthma patients must be treated daily with inhaled corticosteroids instead of as needed because asthma progresses in severity.[44, 48, 50] When patients present uncontrolled asthma, these treatments are not enough to control the symptoms. Fortunately, during the last years the treatment of those patients has improved due to the introduction of new biological agents such as mAb.[51] Omalizumab (anti-IgE) was the first mAb approved for asthma treatment, although others against IL-5 (mepolizumab/reslizumab), IL-5Rα (benralizumab) and IL-4Rα (dupilumab) have been already authorised.[47, 52] These new biologicals together with the advances in serum biomarkers, have improved the phenotype-specific interventions and personalized medicine for asthma.[46]

# 2. Allergy treatments

## 2.1. Allergen-specific immunotherapy (AIT)

Up to date, AIT is the only treatment with potential long-lasting disease-modifying effects for allergic diseases.[53] It consists in the administration of high amounts of specific allergens responsible for the hypersensitivity reaction of the patient to induce a state of permanent tolerance after treatment discontinuation.[53, 54] Currently, AIT is administered either subcutaneously or sublingually to restore healthy immune responses to allergens.[25, 55, 56]

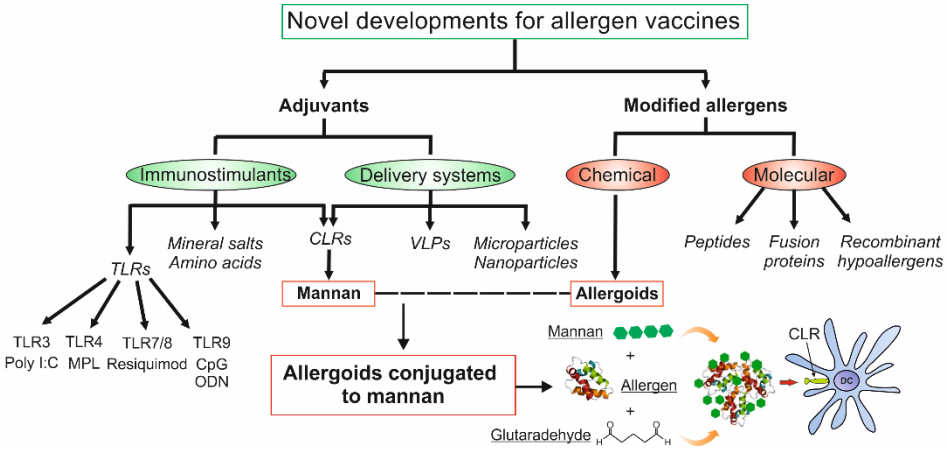


**Figure I.4. Novel developments for allergen vaccines**. Adjuvants can be classified as immunostimulant substances including TLR-ligands, mineral salts, amino acids or CLR-ligands, and as delivery systems such as micro- or nanoparticles, VLPs or CLR-ligands. An allergen can be modified by molecular or chemical strategies. Adjuvants and chemically modified allergens are used in combination for the generation of allergoids conjugated to mannan. *TLR* Toll-like receptor, *CLR* C-type lectin receptor, *VLPs* Viral-like particle, *MPL* Monophosphoryl lipid A, *ODN* Oligodeoxynucleotide. Adapted from *Novel vaccines targeting dendritic cells by coupling allergoids with mannan. Allergo J Int. 2018;27(8):256-262.*

Immunotherapy is indicated for patients with allergic rhinoconjunctivitis, hymenoptera sensitivity or allergic asthma in controlled patients.[57-59] Clinically, the desensitization induced by AIT can translate into long-term allergen-specific tolerance and clinical benefit, lasting for 2 to 3 years after the end of 3 years of AIT treatment.[41] AIT treatment has improved over the last years, but it still has drawbacks in terms of efficacy, security, long duration, or patient compliance. Recent investigations aim to increase efficacy and security of treatments while reducing their duration using, for instance, hypoallergens, new adjuvants or alternative routes of administration (**Figure I.4**).[53, 60-62]

### *2.1.1. Mechanisms in AIT*

The immunoregulatory mechanisms involved in the induction of allergen tolerance have been deeply studied both in patients receiving AIT and in healthy individuals who are naturally exposed to high-doses of allergens, such as beekeepers or cat owners.[25, 63] The disease-modifying effects of AIT are associated with immune modulation of both innate and adaptive immune responses.[62] Successful AIT leading to peripheral tolerance to allergens is associated to a very rapid desensitization of mast cells and basophils and apoptosis of Th2 cells. Afterwards, tolerance induction to allergen requires the inhibition of type 2 immunity-mediated responses, favouring the differentiation into Th1 cells, and the generation and preservation of allergen-specific functional regulatory T (Treg) and B (Breg) cells (**Figure I.5**).[41]

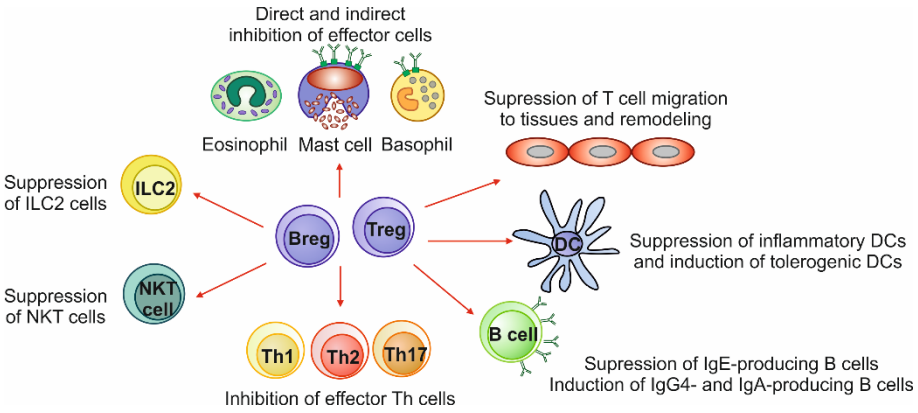


**Figure I.5. Tolerance induced by Treg and Breg cells during allergen-specific immunotherapy (AIT).** Treg and Breg cells supress allergic reactions by inhibiting different crucial inflammatory events. *DC* Dendritic cell, *Th2* T helper type 2 cell, *ILC2* Type 2 innate lymphoid cell, *NKT* Natural killer T cell, *Treg* Regulatory T cell, *Breg* Regulatory B cell.

Allergen-specific $IgG^+/IgA^+$ plasma cells produce blocking antibodies that compete with IgE for the binding to the allergen and can prevent FcεRI-mediated IgE

responses on the surface of mast cells and basophils, inhibiting their degranulation. Besides, blocking antibodies inhibit IgE-facilitated allergen presentation by DCs and B cells and their effects on T cell activation.[20] Upon AIT, $IgE^+$ B cells switch isotype and start producing blocking antibodies with the same allergen-specificity.[41, 64]

Induction of allergen-specific Treg cells is one of the key mechanisms to induce tolerance by AIT (**Figure I.5**). There are two main types of Treg cells, thymus-derived $CD4^+$ $CD25^+$ forkhead box P3 $(FOXP3)^+$ Treg cells (tTregs) and peripherally-derived Treg cells (pTregs), which can be divided into (i) $FOXP3^+$ Treg cells, (ii) $FOXP3^-$ IL10-producing Tr1 cells, and (iii) TGF-β-expressing $T_H3$ cells (**Figure I.6A**).[65, 66] Treg cells are characterized by high expression of the α-subunit of the IL-2 receptor (CD25), lack of expression of the IL-7 receptor α-subunit and expression of FOXP3 transcription factor, which is essential for Treg cell development and function.[67, 68] The most important function of FOXP3 is to confer the suppressive ability to Treg cells, for instance, via maintaining a high expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4).[69] Different studies have demonstrated an increase in the two types of Treg cells upon AIT.[41, 70]

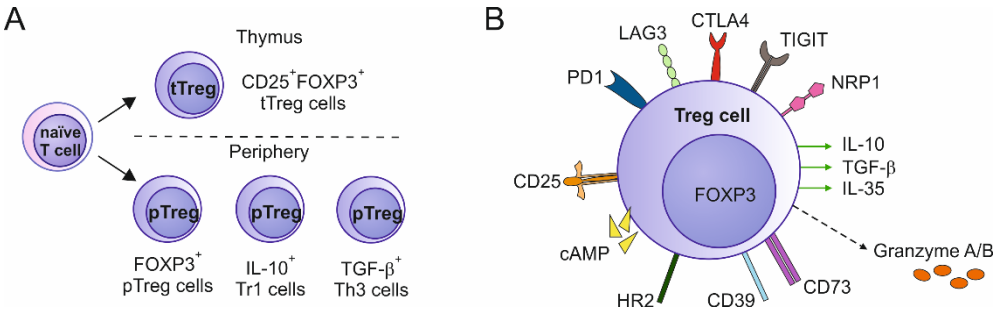


**Figure I.6. Treg cells subsets and suppressive mechanisms. A,** Different subsets of Treg cells, the thymus-derived Treg cells (tTregs) and peripherally-derived Treg cells (pTregs). Adapted from *Regulatory T cells and immune regulation of immune regulation of allergic diseases: roles of IL-10 and TGF-β. Genes Immun. 2014;15(8):511-520.* **B,** Main suppressive mechanisms used by Treg cells: suppressive cytokines (IL-10, TGF-β, and IL-35), metabolic disruption mechanisms (CD25, cAMP, CD39, CD73 and histamine receptor 2 (HR2)), co-inhibitory receptors (such as T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), lymphocyte activation gene 3 (LAG3), programmed cell death 1 (PD1), neuropilin 1 (NRP1) and cytotoxic T lymphocyte antigen 4 (CTLA-4)), and cytolysis by granzymes.

Interestingly, epigenetic modification of FOXP3 promoter region that affects Treg cell function has been observed in AIT, including changes in chromatin accessibility and hypomethylation.[71, 72] In particular, there is a Treg-specific demethylation region (TSDR) within the FOXP3 enhancer CNS2 that is crucial. It has been demonstrated that deletion of CNS2 leads to lower stability and loss of FOXP3 expression in Treg cells.[73] Recent studies have demonstrated that Treg cells display functional adaptability. Treg cells can gain expression of the master transcription factors and chemokine receptors normally associated to Th1, Th2, Th17 and follicular helper T (Tfh) cells, generating Th-like

phenotypes of Treg cells. It has been proposed that this plasticity allows them to migrate to the site of inflammation and inhibit the corresponding T-effector subgroup.[68, 73, 74] The molecular mechanisms associated to the dynamic changes described in Treg cells are not completely understood yet, but it has been shown that the expression of co-inhibitory receptors (or checkpoint receptors) is essential to maintain Treg cell suppressive function and stability. The essential described co-inhibitory receptors include CTLA4, T cell immunoglobulin and mucin domain-containing 3 (TIM3), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), lymphocyte activation gene 3 (LAG3), programmed death 1 (PD1), and neuropilin 1 (NRP1) (**Figure I.6B**).[68] There are four main mechanisms by which Treg cells induce suppression, including suppressive cytokines (IL-10, TGF-β, and IL-35), metabolic disruption mechanisms (CD25, cAMP, histamine receptor 2 (HR2), CD39, and CD73), suppression of DC activation by the co-inhibitory receptors mentioned above, and cytolysis (granzymes A and B) (**Figure I.6B**).

### *2.1.2. Adjuvants in AIT*

Adjuvants are used to induce a quicker, stronger and long-lasting immune response to an allergen vaccine, without being an allergen per se.[75] Proper adjuvants for AIT must be secure, stable and must promote Th1 and/or Treg cell responses.[55, 76] Currently marketed AIT products have 4 different adjuvants: aluminium hydroxide, calcium phosphate, microcrystalline tyrosine (MCT), and monophosphoryl lipid A (MPL).[53, 77, 78] The first three are delivery systems with a depot effect, which means that the adjuvant adsorb the allergen allowing a slow release thus improving tolerability over repeated administrations, although they may also have immunomodulatory properties. On the other side, MPL is a detoxified immunostimulatory agent derived of lipopolysaccharide (LPS) from *Salmonella minnesota* that targets TLR4 in APCs.[78]

Aluminium hydroxide (alum) was introduced as a vaccine adjuvant in 1926 by Glenny *et al*,[79] and since 1937, it is used in AIT. Nowadays, it is the most used adjuvant in AIT.[75, 78] As previously mentioned, for long time the main effects attributed to this adjuvant have been related to its depot effect.[80] Today, several mechanisms have been described. Alum activates innate immune responses by promoting an influx of neutrophils, eosinophils, NK cells, monocytes and DCs to the site of injection and activation of MØ. Alum induces the release of uric acid and double-stranded DNA, which in turn activates NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome and interferon regulatory transcription factor 3 (IRF3), leading to the production of pro-inflammatory cytokines.[81, 82] Besides, alum activates Syk-phosphoinositide 3-kinase (PI3K) pathways and induces prostaglandin E2 production in

MØ and inhibition of IL-12p70 in DCs. Uric acid crystals also activate DCs in a Syk-PI3K-dependent manner, which is involved in Th2 polarization.[81, 82] However, the detailed molecular mechanisms underlying alum adjuvanticity still remain largely unknown.[80]

In addition, intensive research has been done to develop novel adjuvants that can potentiate proper immune responses in target cells (immunostimulants) or increase the capture/presentation of allergens by antigen presenting cells (delivery systems) (**Figure I.4**).[53, 61, 77]

### *2.1.3. Allergoid-mannan conjugates as a novel vaccine for AIT*

The design of novel vaccines targeting DCs has emerged as a useful approach to improve AIT strategies.[60, 83, 84] In this regard, next generation vaccines formulated by the conjugation of allergoids to nonoxidized mannan, which target DCs through their C-type lectin receptors (CLRs) represent novel promising preparations for AIT treatment.[60, 85] Chemical modified allergens (allergoids) are broadly used in AIT because of their safety profile. They display hypoallergenicity, with low reactivity to IgE.[60, 86] Most allergoids are generated by treating the native allergens with aldehydes such as glutaraldehyde or formaldehyde that react with the primary amine groups of lysines. Mannan can be purified from the yeast *Saccharomyces cerevisiae* cell wall keeping the intact mannoprotein associated with the sugar backbone, which contain several lysine residues. Therefore, as the glutaraldehyde is a di-aldehyde capable of reacting with two lysines from different sources, it can crosslink allergen and mannan molecules as glutaryl-diimine groups formed as a result of the reaction (**Figure I.7**). Importantly, this coupling methodology achieves a remarkably stability of the allergoids conjugated to mannan.[61, 86]

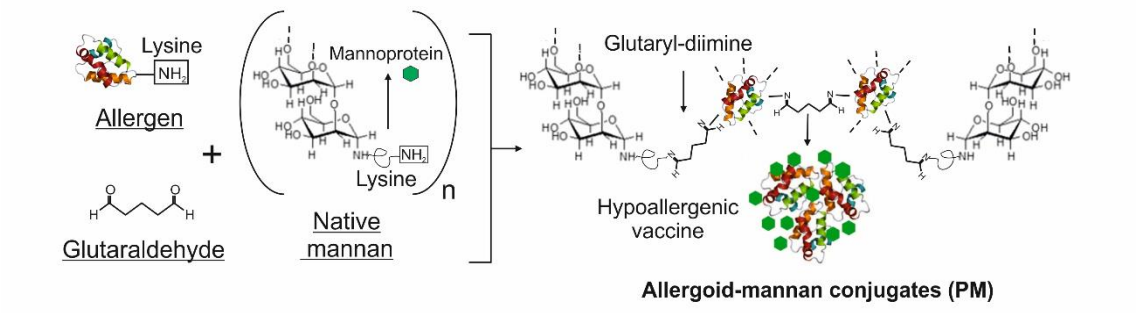


**Figure I.7. Steps for the generation and development of allergoids conjugated to mannan as vaccines for AIT.** Allergens are polymerized and conjugated with mannan in a single step using glutaraldehyde. Adapted from *Novel vaccines targeting dendritic cells by coupling allergoids with mannan. Allergo J Int. 2018;27(8):256-262.*

To assess the potential of these novel allergoid-mannan conjugates to be used as novel AIT vaccines, different preclinical human and animal models were employed. As a proof of concept, allergoids conjugated to nonoxidized mannan from *S. cerevisiae* using *Phleum pratense* grass pollen allergens were produced as shown in **Figure I.7**.

These polymerized allergoid-mannan structures, that will be called PM or allergoid-mannan conjugates from here onwards, are hypoallergenic as they display low *in vitro* IgE binding and reduced capacity to activate *in vivo* mast cells by skin prick test and *ex vivo* basophils from grass pollen allergic patients.[60] Allergoid-mannan conjugates are efficiently captured by human monocyte-derived DCs (hmoDCs), in a process dependent on mannose receptor and specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Remarkably, this enhanced uptake of the vaccine allows to reduce the necessary dose for an efficient response, which might well help to design more effective and shorter immunotherapy protocols.[87] Allergoid-mannan conjugates induce potent IgG blocking antibodies in rabbits and promote the generation of human and mice functional FOXP3$^{+}$ Treg cells through programmed death ligand 1 (PD-L1). In mice, allergoid-mannan conjugates were very efficient increasing IgG2a/IgE and IFN-γ/IL-4 ratios, reflecting a Th1-type driven response (**Figure I.8**).[60, 85]

The first clinical assessment of allergoid-mannan conjugates was performed in dogs with atopic dermatitis, which is the main manifestation of type I hypersensitivity in dogs. They used *Dermatophagoides farinae* allergoids conjugated to mannan administered subcutaneously and observed clinical improvement in most of the cases within the first 3 months of treatment.[88] In humans, four phase II clinical trials have been performed or are currently ongoing for grass pollen, birch pollen, and mite allergy (EudraCT numbers. 2014-005471-88, 2015-000820-27, 2018-002522-23, and 2020-004126-32). Each study is a double dummy with two arms to assess subcutaneous and sublingual administration routes.

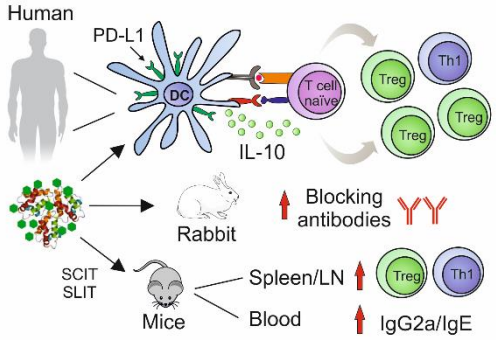


**Figure I.8. Preclinical experiments in human, rabbit and mice to establish the *in vivo* and *in vitro* properties of allergoid-mannan conjugates.** Adapted from *Novel vaccines targeting dendritic cells by coupling allergoids with mannan. Allergo J Int. 2018;27(8):256-262.*

## 2.2. Biologicals

The recent advances in the elucidation of the immunological mechanisms of allergy has allowed the development of novel non-allergen-specific therapeutic strategies which are revolutionizing the treatment of several allergic diseases. Among them, mAbs against specific key targets in the development of allergy stand out.[89-91] Biologicals are usually large molecular-weight therapeutics synthesized by living organisms. In contrast to chemical compounds or small molecules, they have specific targets such as immunoglobulins, cytokines or their receptors, in order to block their action.[92-94] Due to this selectivity, biologicals are ideal for personalized medicine. Biologicals have proven to be efficacious for the treatment of severe allergic asthma, severe eosinophilic asthma, refractory CSU, atopic dermatitis, CRSwNP, and there is increasing evidence for their utility in treating food allergy.[95-97] Therefore, in the context of allergic diseases, many biologicals have been tested, showing efficacy and security.

Currently, the most important are mAbs targeting IgE or type 2 cytokines or their receptors (IL-5 or IL-4/IL-13). For instance, there are 5 biologicals approved for the management of severe allergic asthma and used in the clinical practice, targeted against IgE (omalizumab), IL-4Rα (dupilumab), IL-5 (mepolizumab/reslizumab) and IL-5Rα (benralizumab).[47, 97] Tezepelumab, an anti-TSLP biological is currently in phase III for the treatment of severe asthma (**Figure I.9**).[98]

Other mAbs targeting cytokines implicated in non-type 2 mechanisms (Tumor necrosis factor [TNF]-α, IL-17, Granulocyte monocyte-colony stimulating factor [GM-CSF], etc.) and small molecules (GATA3 transcription factor inhibitors, etc.) are being also tested for allergy and are in different development phases, but none of them is approved yet.[99]

More studies are needed to better understand whether biologicals might have positive disease-modifying effects to prevent the onset or the progression of the disease, the development of multi-morbidities, support the tolerance induced by AIT or create a beneficial effect after stopping the treatment. However, it has not been reported yet that biologicals prevent or modify the course of the allergic disease upon treatment discontinuation.[97]

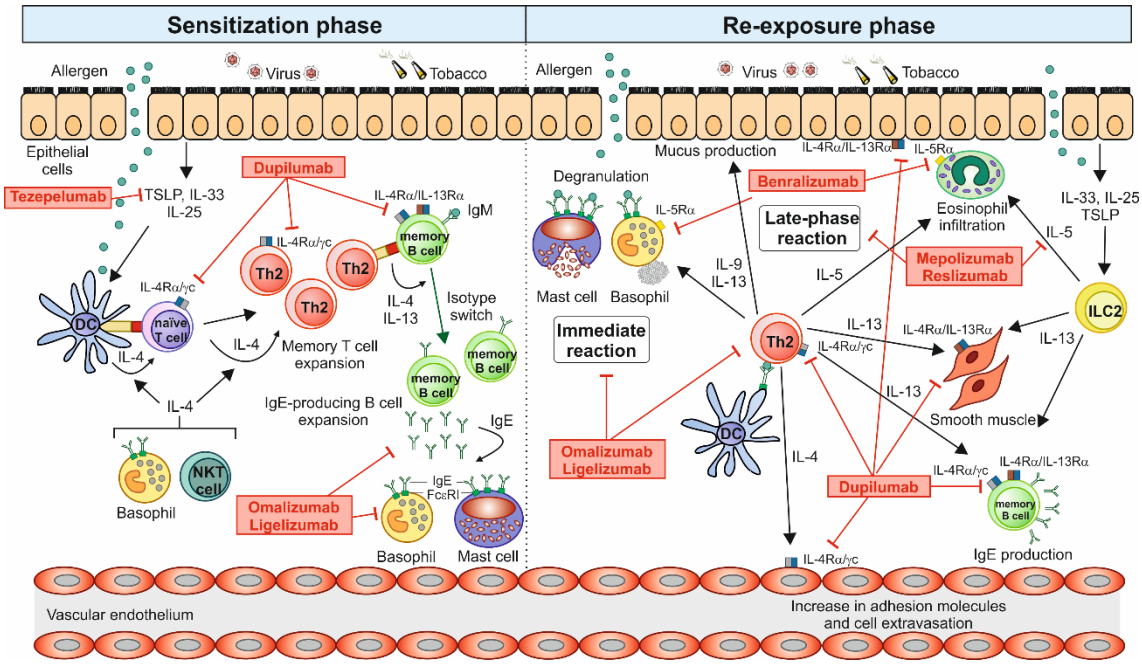


**Figure I.9. Immunological mechanisms involved in allergy and the way of action of mAbs.** Omalizumab/ligelizumab (anti-IgE), mepolizumab/reslizumab (anti-IL-5), benralizumab (anti-IL-5Rα), dupilumab (anti-IL-4Rα), and tezepelumab (anti-TSLP) in the different allergic phases. Adapted from *Diagnóstico, Monitorización y tratamiento inmunológico de las enfermedades alérgicas. 2018, Elsevier.*

### *2.2.1. Anti-IgE treatments*

As mentioned above, IgE plays a key role in allergy. Therefore, the first developed mAb for the treatment of severe allergic asthma specifically targeted IgE.[15] **Omalizumab** is a humanized IgG1 anti-IgE mAb with a molecular weight of approximately 149 kDa that binds with high affinity to IgE at the Cε3 domain with a dissociation constant ($K_D$) of 6-8 nM.[45] Omalizumab interaction site on the Cε3 domain of IgE is different to the interaction site for FcεRI (**Figure I.10**).[36, 100, 101] Omalizumab specifically binds free IgE inhibiting the binding of the antibody to both FcεRI on effector cells, such as basophils, eosinophils and mast cells without inducing crosslinking of the IgE receptors.[101] Besides, its high affinity for IgE allows it to compete with FcεRI.[15] Once bound to free IgE, small complexes are formed, with a size dependent on the relative concentrations of both serum-free IgE and omalizumab. Through reducing serum-free IgE, the expression of FcεRI is

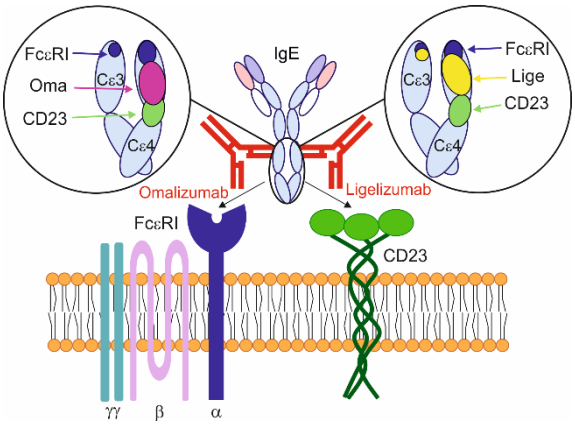


**Figure I.10. Mode of action of anti-IgE mAbs, omalizumab and ligelizumab.** Adapted from *Diagnóstico, Monitorización y tratamiento inmunológico de las enfermedades alérgicas. 2018, Elsevier.*

reduced on mast cells, basophils, and DCs, as well as IgE-CD23 interactions on B cells.[47, 97]

Treatment with omalizumab was approved for the used in 2003 in the USA and was latter approved as adjunctive therapy in 2005 by European Medicines Agency. It is approved for the treatment of severe allergic asthma,[47, 52] severe eosinophilic asthma,[47, 102] CRSwNP,[103] and CSU[91, 104] in adults and children aged 6 years or older.[36, 95]

**Ligelizumab** is a new humanized IgG1 anti-IgE mAb with much higher affinity ($K_D$ of 139 pM) than omalizumab. Ligelizumab and omalizumab recognize distinct epitopes in the IgE Cε3 domain, with some overlap (**Figure I.10**).[101] It has shown dose-dependent and time-dependent suppression of free-IgE, basophil FcεRI, basophil surface IgE, and skin prick test responses to allergens that was superior in extent and duration to those observed with omalizumab.[105] Remarkably, in a phase 2b randomized, double-blind, placebo-controlled clinical trial, it has also been demonstrated that a higher percentage of patients with moderate-to-severe CSU had complete control of symptoms with ligelizumab than omalizumab or placebo.[106, 107]

### *2.2.2. Anti-IL-4/IL-13 treatments*

**Dupilumab** is a human IL-4Rα blocking IgG4 mAb, which inhibits both IL-4/IL-13 through blockade of their shared receptor subunit. IL-4 and IL-13 are structurally and functionally similar cytokines (around 25% sequence identity), composed by four α-helix bundles (**Figure I.11**). IL-4 and IL-13 share many functional characteristics, playing a key role in type 2 responses by promoting Th2 differentiation and IgE production by B cells (only in humans, as IL-13 cannot induce IgE in mice). Both induce airway hyperreactivity, mucus production, smooth muscle cell contraction, and airway remodelling.[94] They are produced by Th2 cells, basophils, mast cells, and NKT cells. ILC2s can also produce IL-13 and small amounts of IL-4. Dupilumab was originally approved for atopic dermatitis, but is now also authorised for severe asthma and severe CRSwNP treatment.[47, 52, 108, 109]

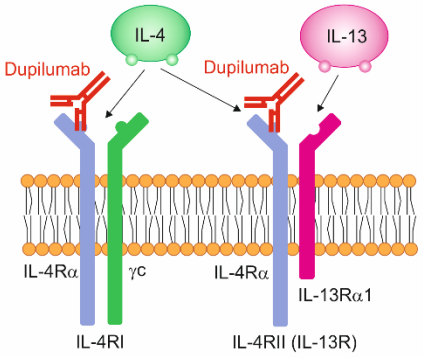


**Figure I.11. Mode of action of anti-IL4Rα mAb, dupilumab.** Adapted from *Diagnóstico, Monitorización y tratamiento inmunológico de las enfermedades alérgicas. 2018, Elsevier.*

### 2.2.3. Anti-IL-5 treatments

**Mepolizumab** and **reslizumab** are humanized IgG1 and IgG4 mAbs, respectively, that specifically target IL-5, inhibiting the activation and recruitment of eosinophils. Similarly, **benralizumab**, another humanized IgG1 mAb, binds the α subunit of the IL-5R, impeding IL-5 signal transduction. Besides, benralizumab binds to FcγRIIIa on natural killer (NK) cells through the Fc region, depleting eosinophils through antibody-dependent cellular cytotoxicity (**Figure I.12**). IL-5 is produced by Th2 cells, mast cells, NKT cells, activated eosinophils, and ILC2s. It binds to IL-5R, a heterodimeric receptor composed of an α subunit responsible for the binding to IL-5 and a common β chain for the signalling. IL-5R is expressed on eosinophils, basophils, mast cells, and B cells. Anti-IL-5 biologicals are efficient against allergic diseases characterized by eosinophilia, such as severe eosinophilic asthma and CRSwNP. Benralizumab is approved for the treatment of severe allergic asthma,[52] and together with mepolizumab and reslizumab, they are approved for severe eosinophilic asthma.[102]

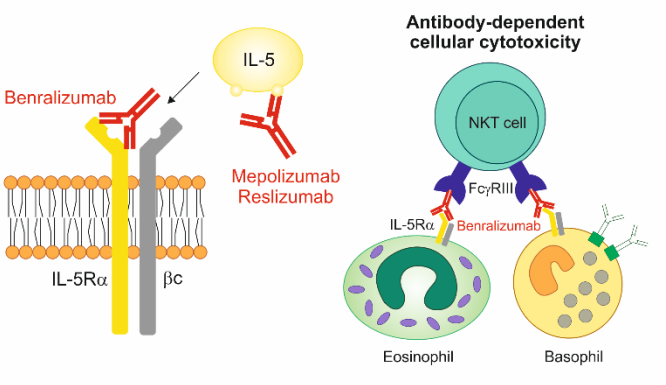


**Figure I.12. Mode of action of anti-IL-5 mAbs.** Mepolizumab/reslizumab (anti-IL-5) and benralizumab (anti-IL-5Rα). The antibody-dependent cellular cytotoxicity reaction is also shown. Adapted from *Diagnóstico, Monitorización y tratamiento inmunológico de las enfermedades alérgicas. 2018, Elsevier.*

# 3. Dendritic cells as therapeutic targets

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

DCs were discovered by Steinman and Cohn in 1973 and are one of the first cell types encountering the allergens in the mucosal surfaces.[110] DCs are the main professional antigen presenting cells linking innate and adaptive immune responses.[111, 112] DCs are located in all peripheral tissues, primary and secondary lymphoid organs and blood.[112, 113] Depending on the encountered antigens and the received signals, DCs display the dual capacity to induce proper immune responses against antigens or to generate tolerance against self-antigens or innocuous molecules.[114-117] To perform their function, DCs display a large number of pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) or CLRs to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Moreover, it has been

proposed the existence of self-PRRs that recognized self-associated molecular patterns (SAMPs) that might well play a role in keeping homeostasis.[118, 119]

As previously described, DCs uptake antigens to present them to naïve T cells.[120] Antigen uptake by DCs is executed by three independent mechanisms: (i) macropinocytosis, which is based on a non-specific uptake of antigens, nutrients, and soluble molecules. (ii) phagocytosis, a process initiated by the activation of surface molecules (although it can also be non-specific) which internalizes large antigens or apoptotic cells by membrane-derived phagosomes, and (iii) receptor-mediated endocytosis, in which specific receptors on the cell surface such as CLRs (i.e., DC-SIGN, mannose receptor), or Fc receptors are involved.[121] Subsequent processing and lysosomal degradation of antigens are crucial, and the most resistant peptides are then presented to the T cell receptor.[121] The antigen presentation is mediated by class I and class II major histocompatibility complex (MHC) molecules, which are expressed on the surface of DCs. Class I MHC molecules bind peptides derived from endogenous antigens and are recognized by $CD8^+$ T cells (cytotoxic). However, class II MHC molecules present peptides derived from exogenous antigens for recognition by $CD4^+$ T cells (helper). Under certain conditions, DCs also have the ability to capture extracellular antigens and present them to $CD8^+$ T cells by class I MHC molecules, a process called cross-presentation.[120, 122, 123]

One of the most critical features of DC biology is their functional maturation, which is a complex process characterized by the acquisition of fundamental properties for the antigen processing and presentation as well as migration and T-cell co-stimulation.[124] In steady-state, DCs are found in peripheral tissues in an immature stage, characterized by reduced expression of mature and co-stimulatory markers CD40, CD80, CD86, and CD83, reduced secretion of cytokines, high phagocytic capacity and limited capacity to stimulate T cells.[125, 126] In response to activation by infection, injury or vaccination, DCs undergo a program of maturation. After this process, DCs are able to migrate to lymph nodes in a process dependent on C-C chemokine receptor 7 (CCR7), and present the processed antigens to naïve T cells to induce T cell differentiation.[127, 128] For long time, it has been accepted that immature DCs mainly induce tolerance through the generation of Treg cells, whereas mature DCs prime different subsets of pro-inflammatory effector T cells depending on the context. However, recent studies have demonstrated that there might be specific conditions in which mature DCs are also able to generate Treg cells.[60, 65]

## 3.2. Dendritic cell subsets

In humans, DCs are derived from $CD34^+$ hematopoietic precursors generated in the bone marrow. DC precursors migrate to the blood and tissues where they generate the main subsets of DCs: conventional or myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).[113] Interestingly, it has been described that FMS-like tyrosine kinase ligand 3 (FLT3L or CD135) is required for the generation and maintenance of DC subsets.[129] Traditionally, DC classification has been based on the expression of surface markers. However, other properties such as the morphology, the migratory capacity and the capacity to stimulate naïve T cells, can help to identify particular DC subsets.[129-131] Although DCs are a heterogeneous population, they are characterized by the expression of class II MHC molecules (HLA-DR) and the absence of linage markers as CD3 (T cells), CD19/20 (B cells) and CD56 (NK cells).[113, 130] Accordingly, human DCs are broadly classified in mDCs and pDCs (**Table I.1**).

**Table I.1**. Phenotype and function of human DC subsets

| | **mDC1** | **mDC2** | **pDCs** | **moDCs** |
|---|---|---|---|---|
| Phenotype | $CD11c^+$, $HLA\text{-}DR^+$, $CD141/BDCA\text{-}3^+$ | $CD11c^+$, $HLA\text{-}DR^+$, $CD1c/BDCA\text{-}1^+$, $CD11b^+$ | $CD11c^-$, $HLA\text{-}DR^{low}$,$CD123^+$, $CD303^+$, $CD304^+$ | $CD1c^+$, $CD11c^+$, $HLA\text{-}DR^+$,$CD11b^+$ |
| PRRs | TLR1, 2, 3, 5, 6, 8 and 10, STING | TLR1-9, RLR, STING | TLR7, 9, RLR, STING | Not well defined |
| Functions | MHCI cross-presentation | MHCII presentation | Anti-viral immunity Tolerance induction | Inflammation |
| Cytokines | Pro-inflammatory | Pro-inflammatory and anti-inflammatory | Antiviral | Pro-inflammatory and anti-inflammatory |
| Equivalent mouse DC subsets | cDC1: $CD11c^+$ $CD8\alpha^+$ (resident) or $CD103^+$ (migratory) | cDC2: $CD11c^+$ $CD11b^+$ $CD1c^+$ | pDCs: $CD11c^{low}$ $B220^+$ $Ly6C^+$ | moDCs: $CD11c^+$ $CD11b^+$ $Ly6C^+$ $CD64^+$ |

### *3.2.1. Human myeloid dendritic cells (mDCs)*

mDCs, also known as conventional DCs, can be defined as $CD11c^+$ and class II MHC $(HLA\text{-}DR)^+$. They were first identified by their morphology and ability to stimulate strong T cell responses, and are the most numerous DC subset in lymphoid organs. mDCs can be divided into two major subsets: $CD141^+$ mDCs (mDC1) or $CD1c^+$ mDCs (mDC2). mDC1 or mDC $CD141^+$ represent around 0.1% of human peripheral blood mononuclear cells (PBMC) and are particularly efficient in the process of cross-presentation. Human mDC1 are the equivalent of mouse conventional DCs (cDC) 1, characterized by expression of CD8α (resident) or CD103 (migratory) (**Table I.1**).[115]

mDC2 or mDC $CD1c^+$ is the most potent subset at driving $CD4^+$ T cell responses and represent around 1% of the whole PBMC.[84] They are located in lymphoid tissues,

skin, lung, and gut and express several PRRs. mDC2 are the human equivalent of the mouse cDC2 (**Table I.1**).[113]

### *3.2.2. Human plasmacytoid dendritic cells (pDCs)*

pDCs represent around 0.2-0.8% of total PBMC and are characterized by the expression of CD123, CD303, CD304 and HLA-DR. pDCs are able to detect exogenous nucleic acids through different intracellular TLRs such as TLR7 and TLR9,[113] and play a key role in viral infections producing high amounts of type I IFN such as IFN-α.[132] Several studies have demonstrated that pDCs from thymus and tonsils can induce $FOXP3^+$ Treg cells with suppressive capacity (**Table I.1**).[133, 134]

### *3.2.3. Human monocyte-derived dendritic cells (hmoDCs)*

Monocytes represent approximately 10% of the whole PBMC and play an important role in innate immune responses against pathogens.[135] *In vitro*, monocytes are differentiated into DCs in the presence of GM-CSF and IL-4.[136] HmoDCs display an immature phenotype with low antigen-presenting capacity. However, under the presence of microbial signals, they are able to capture them and increase their antigen-presenting capacity and induce T cell activation and differentiation (**Table I.1**).[137-139]

## 3.3. Macrophages

MØ are widely distributed in organs and connective tissue, and play central roles in innate and adaptive responses.[140] As DCs, and together with B cells, MØ are professional APCs that can be generated through the differentiation of monocytes.[112] In humans, monocytes migrate into the tissues, especially during inflammatory reactions, and mature into MØ. Tissue MØ perform several important functions:

- Host defense by ingesting and killing microbes.
- Removal of apoptotic host cells. They can recognize and uptake apoptotic cells before they release their contents inducing inflammatory responses.
- Secretion of cytokines when they are activated.
- Antigen presentation, displaying antigens and activating T lymphocytes.
- Promotion of the repair of damaged tissues by inducing new blood vessel growth (angiogenesis) and synthesis of collagen-rich extracellular matrix (fibrosis).

MØ are a very heterogeneous population, displaying the capacity to induce both pro- and anti-inflammatory responses. Classically, the two extremes in the MØ

differentiation are represented by M1-like MØ (or classically activated), which upregulate genes involved in the clearance of pathogens and mediate inflammation in response to extracellular pathogens or M2-like MØ (alternatively activated), which mediate wound healing, clearance of dead and dying cells and tissues, and are involved in anti-inflammatory processes.[141-143] Several studies have summarized the regulation of macrophage polarization by different cytokines, chemokines, and transcription factors.[144-146] LPS, IFN-γ and GM-CSF are potent stimulators of M1-like MØ characterized by high expression of CD80, CD86, CCR7 and production of pro-inflammatory cytokines as TNF-α or IL-6, among others (**Figure I.13**).[147] M2-like MØ can be further divided into M2a, M2b and M2c, according to specific stimulators. M2a are induced by IL-4, IL-13, fungal and helminthic infections; M2b by IL-1R ligands and immune complexes (IC); while M2c are stimulated by IL-10. Phenotypically, they are characterized by expression of CD206, CD162, Arg-1 (**Figure I.13**).[148, 149]

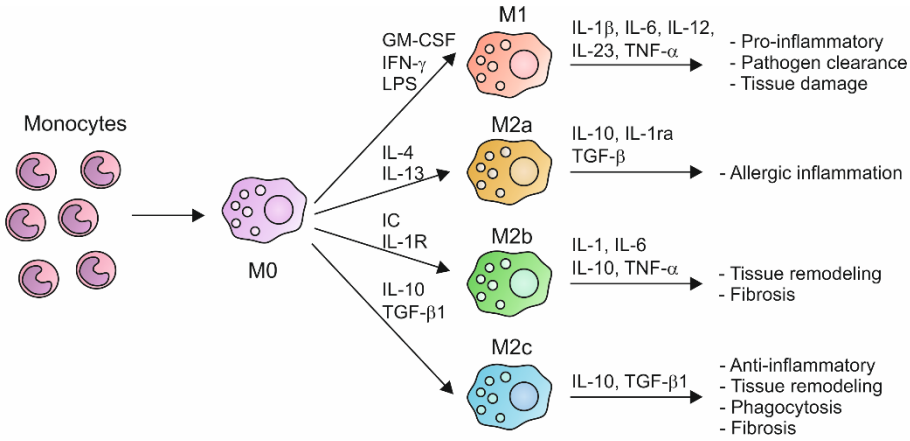


**Figure I.13. Schematic diagram of macrophage subtypes.** M1 subtype is considered pro-inflammatory, M2a subtype is induced by IL-4 and IL-13, which are critical mediators of allergic response. M2b and M2c subtypes are essentially anti-inflammatory and are implicated in tissue remodelling and fibrosis. *IC* Immunocomplexes. Adapted from *Macrophage Polarization and Allergic Asthma. Trans Res. 2017;191:1-14*.

# 4. Immunometabolism

## 4.1. An overview of the main metabolic pathways in the immune system

Metabolic pathways play a major role in immune cell function.[150] Immune cells distinctly use the different metabolic pathways to modulate their function and to produce energy for cell maintenance and proliferation, and modulation of cellular signalling. The glycolysis, tricarboxylic acid (TCA) cycle, pentose phosphate pathway (PPP), fatty acid oxidation, fatty acid synthesis, and amino acid synthesis are the main cellular metabolic pathways involved in immunometabolism (**Figure I.14**):[151]

- **Glycolytic metabolic pathway**. Glycolysis begins with the uptake of extracellular glucose and its subsequent intracellular processing in the cytosol to generate pyruvate along with other products. Pyruvate can then be converted in lactate (lactic acid fermentation) or acetyl coenzyme A to enter in the TCA at the mitochondria. Glycolytic metabolism is a relatively inefficient pathway due to the low generation of ATP (just two ATPs per glucose molecule). However, it represents a fast generation of ATP and also allows the reduction of $NAD^+$ to NADH, used as a cofactor for many other enzymes.[151]
- **TCA cycle and oxidative phosphorylation**. The TCA cycle, also known as the Krebs cycle or citric acid cycle, takes place inside the mitochondria, generally in quiescent or non-proliferative cell settings with requirements of energy and longevity. The TCA cycle receives nutrients from several pathways (as pyruvate from glucose, fatty acids or amino acids). The two major products of TCA are NADH and $FADH_2$, which transfer electrons to the electron transport chain to support oxidative phosphorylation (OXPHOS) generating a highly efficient ATP production in the presence of oxygen.[151]

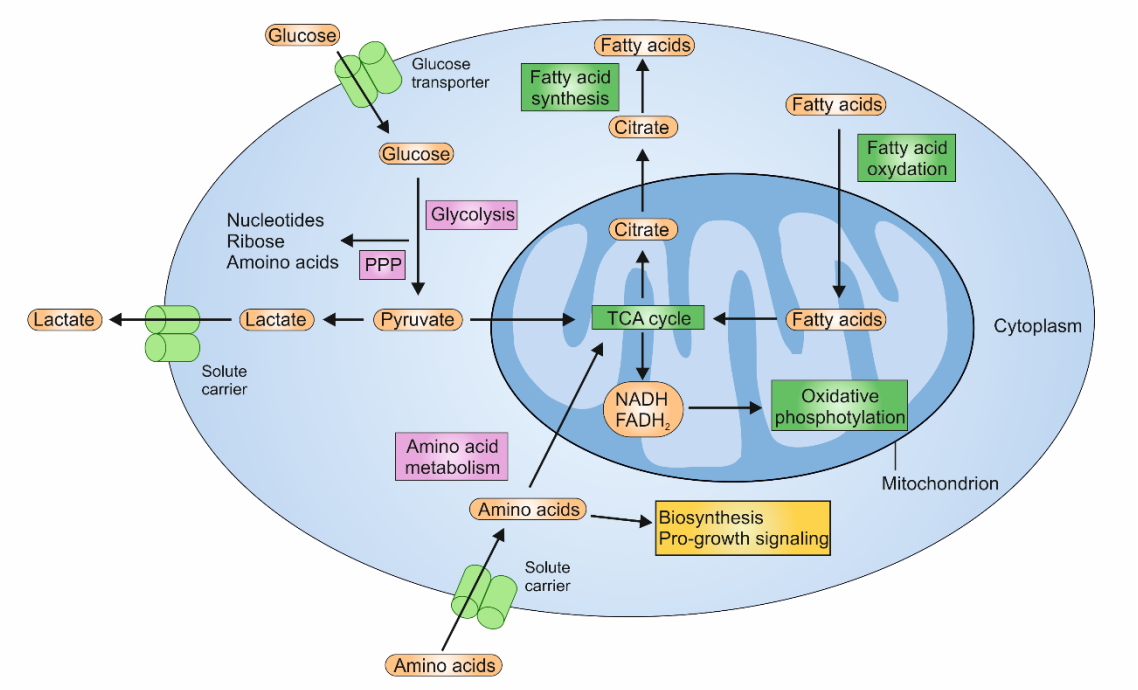


**Figure I.14. Six major metabolic pathways:** glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation, pentose phosphate pathway (PPP), fatty acid synthesis, fatty acid oxidation and amino acid metabolism. In this figure, the pathways that require oxygen are indicated in green boxes, and non-oxygen dependent pathways are indicated in pink boxes. Adapted from *A guide to immunometabolism for immunologists. Nat Rev Immunol. 2016; 16(9):553-565.*

- **Pentose phosphate pathway**. PPP takes place in the cytosol and serves to support cell proliferation and survival. PPP allows the diversion of intermediates from the glycolytic pathway towards the production of nucleotide and amino acid precursors and generates reducing equivalents of NADPH, which play a key role in the maintenance of the cellular redox environment.[151]
- **Fatty acid oxidation**. The fatty acid oxidation of the mitochondrial fatty acids generates high amounts of ATP molecules and products that the cell can further use to produce energy, including Acetyl-CoA, NADH, and $FADH_2$.[151]
- **Fatty acid synthesis.** The fatty acid synthesis allows cells to generate lipids necessary for cellular growth and proliferation from precursors from other metabolic pathways. The mammalian target of rapamycin (mTOR) signalling is closely related to this fatty acid synthesis.[151]
- **Amino acid synthesis.** Amino acids, as substrates for protein synthesis, are linked to anabolic signalling pathways. Individual amino acids play specific roles in metabolic pathways. For instance, arginine and tryptophan are metabolized to support cellular proliferation and anabolic growth.[151]

## 4.2. Metabolic control of dendritic cell function

The understanding of the cellular metabolism influencing the ability of DCs to prime immunity or induce tolerance is an emerging fascinating field with active research over the last years.[150, 152, 153] In living organisms, cellular metabolism is critical for the production of energy in the form of ATP as well as for proliferation and cell maintenance.

The change from resting to activated-DCs involves a transition in which the cells display more dendrites and become more secretory and interactive with other cells. These changes in DC biology are accompanied with changes in cellular metabolism.[154] In resting DCs, the anabolic demands are low, and the catabolism of proteins and triacylglycerols provides substrates for ATP production by TCA cycle and OXPHOS.[153-155] DC activation in response to innate sensing of pathogens induces a higher consumption of glucose and lactic acid production, in a phenomenon similar to Warburg effect.[156] Warburg effect, described by Otto Warburg, is the change in metabolism towards glycolysis with high lactate production despite the availability of oxygen (**Figure I.15**).[152] This shift in cell metabolism can be explained by the quick requirements of energy for biosynthesis in contrast to an efficient generation of energy.

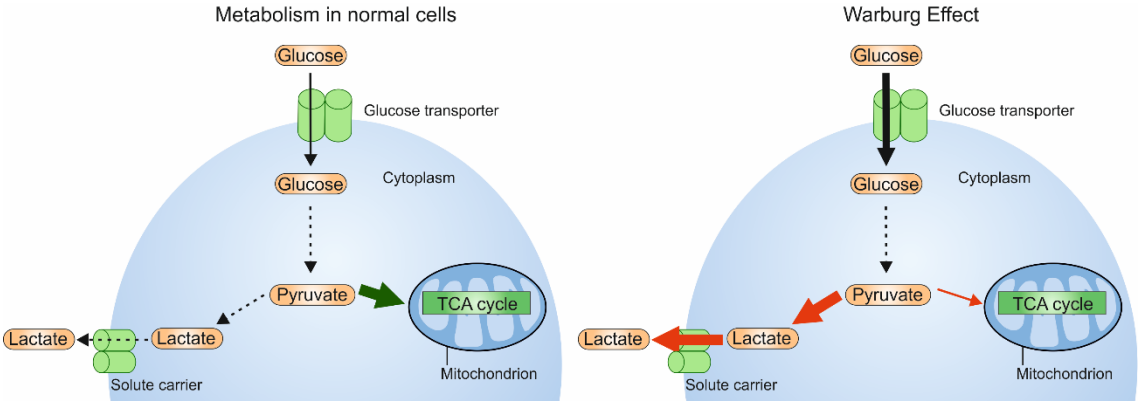


**Figure I.15. Warburg effect in immune cells**. The change in metabolism towards glycolysis with high lactate production despite the availability of oxygen is known as the Warburg effect. *TCA* Tricarboxylic acid.

Studies about the metabolic state of several immune cells have been performed over the last years. For instance, it is known that in T cells, metabolism defines the immune response. Memory T cells are dependent on fatty acid oxidation and glucose and, generally, glycolysis fuels pro-inflammatory effector T cells.[157, 158] Regarding Treg cells metabolism, while glycolysis is essential for their proliferation and expansion, the suppressive capacity is mainly maintain by oxidative metabolism.[158] Although there is still a limited knowledge about how the metabolism of DCs is affected by the environmental signals and how its changes can affect DC functions, some studies suggest that moDCs and cDC1 generation is more dependent on functional mitochondrial metabolism and OXPHOS than cDC2 or pDCs.[153] The importance of metabolic control was first assessed in the development of moDCs *in vitro*. HmoDCs survival and differentiation rely on mTOR complex I activation via PI3K.[159] It has been described that moDCs differentiation depends on OXPHOS and a balanced fatty acid metabolism.[153] Similarly, resting and activated DCs show differences in their metabolic priorities. Maturation of DCs by different TLR stimuli activates signalling cascades that determine the metabolic regulation.[160]

Tolerogenic DCs (tol-DCs) also show a characteristic metabolic profile. In recent studies, Malinarich *et al* have described that the expression of metabolic pathway-related genes shows differences within DCs.[161] They demonstrate that there is an increased expression of genes related to OXPHOS in tol-DCs, especially of electron transport chain complexes II and IV. Moreover, IL-10 shifts the metabolism toward a higher OXPHOS and lower glycolysis after TLR stimulation. However, tol-DCs can also display enhanced glycolytic capacity and reactive oxygen species (ROS) production with respect to mature pro-inflammatory DCs.[161, 162] The catabolic profile and the high-energy demand observed in tol-DCs may be related to their active suppressive function.[161, 163]

Recent studies show a complex and continuous interplay between immunometabolism and epigenetics. Different metabolic pathways serve as a source of energy for the active epigenetic remodelling in the cells as well as a source of substrates to modify the structure of the different regions of the histones and genome.[164, 165]

# 5. Epigenetics

## 5.1. An overview of the main epigenetic modifications

Epigenetics is defined as a stably heritable phenotype resulting from changes in a chromosome without changes in the DNA sequence. The term epigenetic is, however, increasingly taken to include transient chromatin modifications as long as they result in altered gene transcription.[166] Precise control of gene expression is achieved by epigenetic mechanisms, which facilitate heritable and stable programming of gene transcription while retaining the potential to be modified. Epigenetic changes play a pivotal role in the adaptation of the transcriptional response. Epigenetic mechanisms regulate cell development, identity, and function. Mechanisms mainly include histone post-translational modifications, DNA methylation and non-coding microRNAs (miRNAs) (**Figure I.16**).

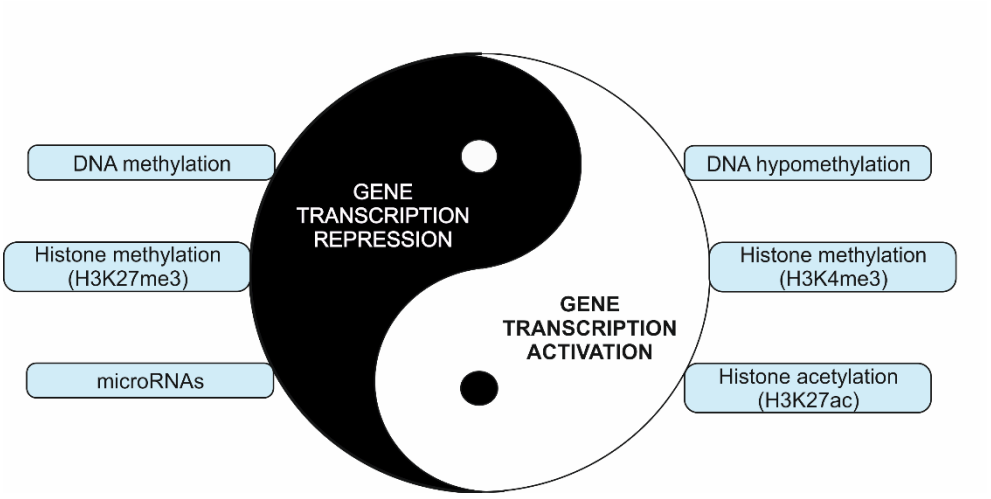


**Figure I.16. Main epigenetic mechanisms and their effect on gene transcription**. DNA methylation, histone methylation in specific sites as the lysine 27 of the histone 3, and microRNAs induce gene transcription repression. On the contrary, DNA hypomethylation, histone methylation in specific sites as the lysine 4 of the histone 3, and histone acetylation induce gene transcription activation.

Histone modification, as an epigenetic mechanism, including acetylation, methylation, phosphorylation, deamination, β-*N*-acetylglucosamine, ADP ribosylation, ubiquitination, and sumoylation of histones, can change the charge of histones which subsequently affect the structure of chromatin to upregulate or downregulate gene

expression (**Figure I.16**).[167, 168] Distributions of histone 3 lysine 4 trimethylation (H3K4me3), histone 3 lysine 27 trimethylation (H3K27me3) or histone 3 lysine 27 acetylation (H3K27ac) in promoter regions of genes can be assessed by chromatin immunoprecipitation (ChIP) assays.[167] This modifications are executed by histone methyltransferases (HMTs), histone acetyltransferases (HATs) and histone deacetylases (HDACs), among others. The main histone modifications in atopy and allergic diseases and their effects on gene transcription are listed on **Table I.2**.[168]

**Table I.2.** List of the main histone modifications described for atopy and allergic diseases.

| Modification and site | Abbreviation | Effect on transcription |
|---|---|---|
| Histone acetylation | | |
| Histone 3 panacetylation | H3ac | Activating |
| Histone 4 panacetylation | H4ac | Activating |
| Histone 3 lysine 4 acetylation | H3K4ac | Activating |
| Histone 3 lysine 9 acetylation | H3K9ac | Activating |
| Histone 3 lysine 14 acetylation | H3K14ac | Activating |
| Histone 3 lysine 27 acetylation | H3K27ac | Activating |
| Histone 4 lysine 16 acetylation | H4K16ac | Activating |
| Histone methylation | | |
| Histone 3 lysine 4 methylation | H3K4me1 | Activating |
| Histone 3 lysine 4 dimethylation | H3K4me2 | Activating |
| Histone 3 lysine 4 trimethylation | H3K4me3 | Activating |
| Histone 3 lysine 9 dimethylation | H3K9me2 | Repressive |
| Histone 3 lysine 9 trimethylation | H3K9me3 | Repressive |
| Histone 3 lysine 27 trimethylation | H3K27me3 | Repressive |
| Histone 3 lysine 36 trimethylation | H3K36me3 | Activating |

DNA methylation is a heritable epigenetic mark involving the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs). DNMT3A and 3B are the major the novo methyltransferases while DNMT1 maintain DNA methylation upon DNA replication.[169] DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factor(s) to DNA.[170] DNA methylation and histone post-translational modifications act together to establish an open or closed chromatin structure, which define active or inactive gene transcription. In general, DNA hypomethylation is associated with active chromatin while histone marks are known to work in a synergistic way to activate or repress genes.[171, 172]

MiRNAs are small endogenous single-stranded RNAs of approximately 21-24 nucleotides that post-transcriptionally modulate gene expression and have important biologic functions. They regulate gene expression through targeting the 3'-UTR of mRNA, resulting in either translational repression or mRNA degradation, or both.[173, 174]

## 5.2. Epigenetic control of dendritic cell function

Recent evidences show that epigenetic alterations as DNA methylation, histone modifications or miRNAs, are involved during differentiation of hmoDCs and in their dual capacity to become activated or tolerogenic cells.[175, 176] The differentiation of monocytes into DCs implies the downregulation of CD14 and upregulation of CD209 (or DC-SIGN), an essential molecule for the trafficking and contact with T cells. This process is mediated by the acquisition of the histone 3 lysine 9 acetylation (H3K9ac) active histone mark in CD209 together with the loss of repressive marks, histone 3 lysine 9 trimethylation (H3K9me3) and histone 4 lysine 20 trimethylation (H4K20me3), and loss of DNA methylation.[177] Besides, there is a balance between the repressive H3K27me3 and active H3K4me3 histone marks in genes that modulate their expression and regulate the differentiation and the activation of hmoDCs.[178]

Epigenetic modifiers, such as HDAC inhibitors, can also affect DC functions. It has been demonstrated that HDAC inhibition reduces the expression of costimulatory molecules such as CD40, CD80, and CD86 both in human and mouse immature and mature DCs, and increases the expression of indoleamine 2,3-dioxygenase (IDO) in murine DCs.[179] Moreover, HDAC inhibitors reduce allergic inflammation and therefore could be used as a potential anti-inflammatory strategy for the treatment of allergic diseases.[180-182] DNA methylation has been reported to regulate the cytokine pathway of IL-4 by DCs as well as T helper differentiation. However, the exact mechanisms remain unknown.[183]

miRNAs also play a central role in the generation of human tol-DCs during monocyte differentiation. Most immune cells such as DCs and MØ display specific miRNAs signatures that are essential to maintain gene regulation. These miRNAs change their expression during cell stimulation or differentiation.[184, 185] Up to date, several miRNAs have been identified for their involvement in DCs development.[174] Among them, some display specific relevance for the generation of tolerance. For example, miRNA-155 acts primarily as a pro-inflammatory factor. The upregulation of miRNA-155 during inflammation processes has been correlated with the hyperactivation of myeloid cells, while inhibition of miRNA-155 increases the expression of suppressor of cytokine

signalling (SOCS) 3.[186] MiRNA-146a/b are known to inhibit NF-κB pathway among others, with an anti-inflammatory role, and miRNA-21 inhibits inflammatory responses by targeting tumour suppressors and a pro-inflammatory protein (Programmed Cell Death Protein 4, PDCD4) that promotes the activation and contributes to Th2 cells.[187] Further investigations are still needed to improve our knowledge about the role of epigenetic modifications on the control of DC function.

# OBJECTIVES

The increasing prevalence of allergic diseases represents a very serious health problem with a high socioeconomic burden. A detailed understanding of the immunological mechanisms that underlie allergy as well as the processes involved in the generation and maintenance of allergen tolerance, are key for the development of novel strategies to treat and prevent allergic diseases. Currently, there are useful treatments that have significantly improved the quality of life of many patients suffering from allergic diseases. Among them, allergen-specific immunotherapy (AIT) and biologicals targeting key molecules driving allergy such as IgE, represent the most effective and advanced therapies used in the clinic. However, there are still several important drawbacks associated to these treatments, including safety, efficacy, low adherence, high cost, long term, and side effects that require further investigations.

We hypothesize that the better understanding of the mode of action of AIT and biological treatments might help us to increase the knowledge of the molecular mechanisms underlying tolerance and allergy as well as to design better therapies in the future. At this regard, dendritic cells (DCs) play a central role both in the development of allergy and in tolerance induction, representing the link between innate and adaptive immune system. Considering all these aspects, the global aim of this Doctoral Thesis is to study the molecular mechanisms by which next generation AIT vaccines (allergoid-mannan conjugates) and anti-IgE biologicals (omalizumab and ligelizumab) might influence DC's function and their capacity to generate functional regulatory T (Treg) cells.

To achieve this main objective, we will pursue the following specific aims encompassed in two different blocks:

**Block A**: Study of the molecular mechanisms involved in the mode of action of next-generation vaccines for AIT targeting DCs.

The main aim of this block is to study the immunological mechanisms by which polymerized allergoids conjugated to mannan induce the generation of tolerogenic DCs able to promote Treg cells.

Objective 1. Study of the potential influence of aluminium hydroxide in the tolerogenic properties imprinted by allergoid-mannan conjugates at the molecular level (Article I).

1.1. To study the effect of the presence of aluminium hydroxide in the tolerogenic properties imprinted by allergoid-mannan conjugates in DCs.

1.2. To study the generation of Treg cells induced by DCs stimulated with allergoid-mannan conjugates previously adsorbed to aluminium hydroxide.

1.3. To study the molecular mechanisms implicated in the influence of aluminium hydroxide in the effects induced by allergoid-mannan conjugates in DCs.

Objective 2. Study of the capacity of allergoid-mannan conjugates to reprogram monocyte differentiation into tolerogenic DCs and macrophages and the elucidation of the underlying molecular mechanisms (Articles II and III).

2.1. To study whether allergoid-mannan conjugates could reprogram monocyte differentiation into tolerogenic DCs.
2.2. To study the molecular mechanisms underlying the reprogramming of monocytes into tolerogenic DCs.
2.3. To study whether allergoid-mannan conjugates could reprogram monocyte differentiation into tolerogenic macrophages.

**Block B**: Study of the capacity of anti-IgE biologicals to immunomodulate the ability of DCs to promote the generation of Treg cells.

The main aim of this block is to study the influence of biologicals targeting IgE (omalizumab and ligelizumab) to condition the capacity of DCs to polarize Treg cells as well as the molecular mechanisms involved in such effects.

Objective 3. Assess the capacity of omalizumab, an anti-IgE mAb, to promote the induction of functional Treg cells in comparison to corticosteroids (Article IV and Annex I, unpublished).

3.1. To study the immunomodulation exerted by omalizumab and corticosteroids on the phenotypic and functional properties of human DCs.
3.2. To study the capacity of omalizumab to condition the capacity of DCs to polarize T cell responses in comparison to corticosteroids.
3.3. To study the molecular mechanisms by which omalizumab condition the capacity of human DCs to generate Treg cells.

Objective 4. Assess the capacity of ligelizumab, a new anti-IgE mAb, to promote the induction of functional Treg cells in comparison to omalizumab (Annex II, unpublished).

4.1. To study the immunomodulation exerted by ligelizumab compared to omalizumab on the phenotypic and functional properties of human DCs.
4.2. To study the capacity of ligelizumab compared to omalizumab to condition the capacity of DCs to polarize T cell responses, with special focus on the generation of Treg cells.

# MATERIALS AND METHODS

## 1. Allergen-specific immunotherapy vaccines

Glutaraldehyde-polymerized grass pollen (*Phleum pratense)* allergoids conjugated to nonoxidized mannan, native grass pollen *P pratense* allergens and mannan-free glutaraldehyde-polymerized allergoids were provided by Inmunotek SL. Defatted grass pollen grains from *P pratense* (Iberpolen, Jaén, Spain) were extracted overnight with PBS (pH 7.2) and subjected to tangential flow ultrafiltration (cut-off pore size, 100 kDa). The enriched native grass pollen *P pratense* allergens (N) contained in the filtrate were dialyzed with distilled water and lyophilized until use. Total protein content was measured by using the Bradford assay with serum albumin as the standard (Bio-Rad Laboratories). The native mannan (nonoxidized) was purified from the yeast *Saccharomyces cerevisiae* (Lesaffre), according to previously described protocols with slight modifications.[86] N were polymerized and conjugated with mannan through the lysine residues from its mannoprotein, as previously described.[60, 86] Briefly, glutaraldehyde (25 mmol/L) was added to a solution containing a mixture of allergen and mannan (1:0.5 wt/wt) in PBS for 6 hours at 4ºC in continuous stirring. The reaction was stopped with glycine (1.25 mol/L), followed by tangential flow filtration with distilled water (membrane cut-off, 100 kDa). Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan (PM) were recovered in the concentrated retained fraction (>100 kDa). The same native allergen extract was subjected to the above protocol but without mannan to obtain mannan-free glutaraldehyde-polymerized allergoids (P).

(For Articles I, II, and III)

## 2. Allergic patients and nonatopic donors

We obtained buffy coats from Centro de transfusiones de la Comunidad Autónoma de Madrid (Valdebernardo, Madrid). Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats and specific IgE against grass pollen and other common aeroallergens and total serum IgE levels were measured. Donors were considered nonatopic when negative for specific IgE and with serum IgE levels <30 ng/mL, as determined by ELISA. Besides, fresh blood from grass pollen-allergic patients has been used for specific experiments, whose demographic and clinical features are collected in **Table M.3**. Those patients had recurrent clinical symptoms during the grass pollen season, displayed specific IgE against grass pollen allergens and were previously clinically characterized.

(For Articles I, II, III, IV, and Annexes I, II)

**Table M.3.** Clinical features of patients with grass pollen allergy

| Patient no. | Age (y) | Sex | Allergic to | Specific IgE(1) | Symptoms | Other pollen allergies |
|---|---|---|---|---|---|---|
| 1 | 28 | M | Grasses | 3.220 | RC | *Olea* species, *Cupressus* species |
| 2 | 26 | F | Grasses | 0.355 | RC | *Olea* species, *Cupressus* species |
| 3 | 30 | M | Grasses | 0.603 | RC | |
| 4 | 27 | F | Grasses | 3.500 | RC | *Olea* species, *Platanus* species |
| 5 | 57 | M | Grasses | 0.642 | RC-A | *Olea* species, *Cupressus* species |
| 6 | 27 | M | Grasses | 1.300 | RC-A | *Platanus* species, *Betula* species |

*A*, Asthma; *F,* Female*; M,* Male*; RC*, Rhinoconjunctivitis;

(1): Determined in ELISA as OD at 492 nm

## 3. Material, media and reagents

For cell cultures, we used RPMI 1640 (Lonza) supplemented with 10% heat-inactivated foetal bovine serum, 100 µg/mL normocin (InvivoGen), 50 µg/mL penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mM sodium pyruvate (Life Technologies). For experiments with THP1 cells, also glucose up to 4.5 g/L was added.

Aluminium hydroxide gel (Alhydrogel) was from InvivoGen. Inhibitors for mTOR (rapamycin) (InvivoGen), ROS (N-acetyl-cysteine (NAC)) or glycolysis (2-Deoxy-D-glucose (2-DG)) (Sigma-Aldrich) were used for the inhibition experiments.

Lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 and inhibitors for histone methyltransferase (MTA) and histone demethylase (pargyline) (Sigma-Aldrich) were used in cell cultures. Mannan (Sigma-Aldrich) was used to differentiate monocytes into DCs for comparing experiments.

For pDCs experiments, recombinant human IL-3 (10 ng/mL, Peprotech) was added to the culture media in all the experiments. pDCs were stimulated with CpG class B TLR9-ligand (ODN 2006, 2 µM, InvivoGen), rabbit anti-human IgE (IgE-FcεR1-crosslinker, IgE-CL, 10 µg/mL) and rabbit whole IgG control antibody (IgG, both from Bethyl Laboratories). Human IgE monoclonal antibody (BioPorto Diagnostics) or chimeric human IgE JW8 and NIP-BSA (both from NBS BioScience), recombinant human TNF-α (10 ng/mL, PreproTech) and IFN-$\alpha_2$ (IFN-α, 5 ng/mL, Biolegend), L-kynurenine (kyn, 0.5 µM, Sigma-Aldrich), neutralizing antibodies anti-human IFN-α/β receptor (anti-IFNAR, 5 µg/mL) (clone MMHAR-2, EMD Millipore), anti-human TNF-α (clone MAb11, 5 µg/mL,, Biolegend) and 1-Metil-L-Tryptophan (1-MT, 250 µmol/L,

Sigma-Aldrich) were used. Omalizumab (Xolair) and Ligelizumab were from Novartis Pharma AG, and Dexamethasone (1 µM) was from Sigma-Aldrich. For lactic acid stripping buffer, we used L-(+)-lactic acid 0.01 M, NaCl 0.13 M, KCl 0.005 M (pH 3.9). DARPin bi53-79 was kindly provided by Alexander Eggel from University Hospital Bern, Switzerland, under a proper material transfer agreement between Universities.

(For Articles I, II, III, IV, and Annexes I, II)

# 4. Isolation of different cell subsets

## 4.1. Isolation of peripheral blood mononuclear cells

Human PBMCs were obtained from buffy coats or fresh blood of nonatopic donors or allergic patients by Ficoll density gradient centrifugation. The centrifugation conditions were 800g during 20 minutes at room temperature. After centrifugation, the white interface containing the PBMC fraction was collected and washed with PBS. Finally, viable cells were counted with Trypan blue in a Neubauer chamber under a light microscope. The different cell populations obtained from PBMCs are represented in **Figure M.17**.

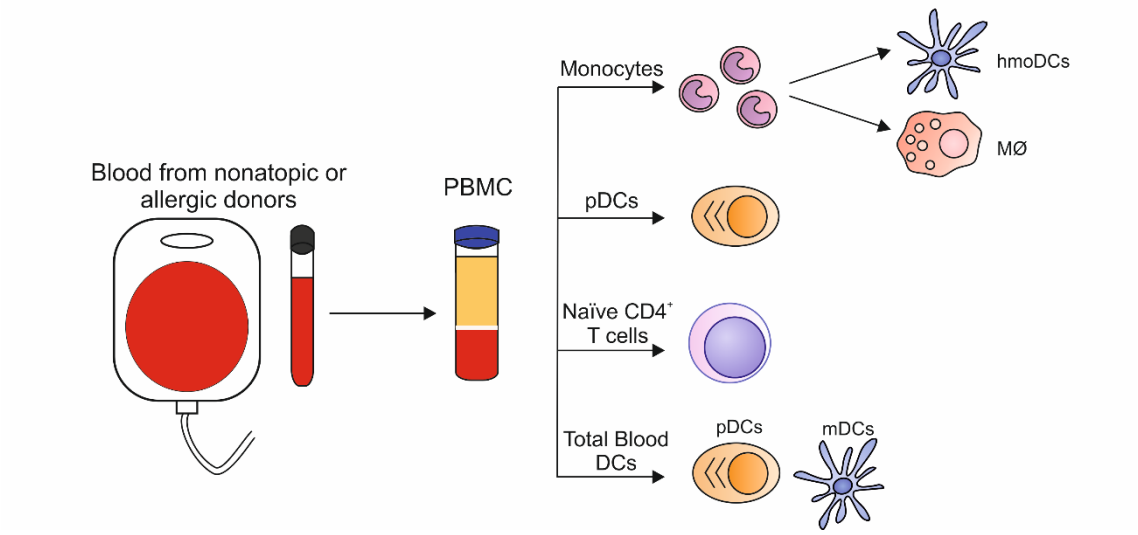


**Figure M.17. Purification of different cell populations from PBMCs from nonatopic or allergic donors.** PBMCs isolated from peripheral blood from buffy coats or conventional blood extraction are used to purify total blood DCs containing plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), naïve $CD4^+$ T cells, pDCs, and monocytes for their differentiation into hmoDCs or MØ.

## 4.2. Purification of monocytes

Human blood monocytes were obtained from total PBMCs using antibodies against human CD14 coupled to microbeads (Miltenyi Biotec) and using the AutoMACS Pro

system (Miltenyi Biotec) through positive selection according to manufacturer's instructions.

(For Articles I, II, III, IV, and Annex I)

## 4.3. Generation of human monocyte derived DCs (hmoDCs)

The purified monocytes were then differentiated into immature hmoDCs. Monocytes were cultured in 24-well plates at a concentration of $1x10^6$ cells/mL with complete RPMI medium containing 100 ng/mL of IL-4 and GM-CSF (PeproTeck). They were incubated for 6 days at 37ºC in a humidified 5% $CO_2$ incubator. To generate hmoDCs differentiated in the presence of PM (mannan-tolDCs), 50 µg/mL of PM was added at days 0 and 4 of the differentiation. 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).[137, 138]

(For Articles I, II, III, and Annex I)

## 4.4. Generation of human macrophages

To generate human monocyte-derived MØ, monocytes were cultured at $0.5x10^6$ cells/mL in 48-well microplates for 6 days with complete RPMI medium containing GM-CSF (100 ng/mL, GM-MØ) or M-CSF (10 ng/mL, PeproTeck, M-MØ). Addition of cytokine took place every 2 days. To generate Mannan-MØ, 50 µg/mL of PM was added at days 0 and 4 of the differentiation. In all the cases, viable cells were counted using Trypan blue with an optical microscope and analysed by propidium iodide by flow cytometry.

(For Article III)

## 4.5. Purification of naïve $CD4^+$ T cells

Naïve $CD4^+$ T cells were purified from PBMCs using "Naïve $CD4^+$ T Cell Isolation Kit" (Miltenyi Biotec), and the AutoMACS Pro (Miltenyi Biotec) according to manufacturer's instructions with minor modifications. The cells were purified through a negative selection, labelling all the rest of the cells with microbeads conjugated with 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 95-99% as checked by flow cytometry with anti-CD45RA, anti-CD4 and anti-CD3 antibodies.[137, 138]

(For Articles I, II, III, IV, and Annexes I, II)

### 4.6. Purification of total blood DCs

Total blood DC fraction was obtained from PBMCs using “Blood Dendritic Cell Isolation Kit II” (Miltenyi Biotec), following the manufacturer’s protocol and using the autoMACS Pro (Miltenyi Biotec). With this kit, both pDCs and mDCs were purified. The enriched fraction had more than 90% of total DCs and was tested with anti-CD1c, anti-CD303, anti-CD19 and anti-HLA-DR at CCMF from UCM.[60, 137]

(For Article I)

### 4.7. Purification of pDCs

Plasmacytoid dendritic cells (pDCs) were isolated (85 – 95% purity) from PBMCs with the “Plasmacytoid dendritic cell isolation kit II” in autoMACS Pro (Miltenyi Biotec). The mean value of the percentage of pDCs displaying IgE bound to FcεRI when considering all the assayed donors was 45.71 ± 6.23 (mean ± SEM). This value was used as a cut-off to differentiate nonatopic vs atopic donors, we selected for further experiments those donors whose pDCs displayed FcεRI-bound IgE frequencies higher than 45% ($IgE^+$ pDCs or atopic donors).

(For Article IV and Annexes I, II)

### 4.8. Generation of THP-1 macrophages

THP1 cells, which are a human monocytic cell line, were grown in culture medium. Prior to the experiments, cells were differentiated to MØ by incubation with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in culture medium for 24 hours. They were then washed with medium and grown in culture medium for 24 hours. Then, THP1 MØ were polarized by incubation for 18 hours with 50 ng/mL IFNγ (Preprotech) plus 100 ng/mL LPS (IFN/LPS), 40 ng/mL IL4 (Preprotech), 50 ng/mL IL10 (Preprotech) or 50 µg/mL of PM. The control population was incubated in culture medium (Ctrl-) without polarizing cytokines. Subsequently, cells were collected, centrifuged and used for phenotype characterization by quantitative real-time RT-PCR (qPCR) analysis.

(For Article III)

## 5. Cell culture procedures

### 5.1. Culture of human DCs

Immature hmoDCs or human total blood DCs from healthy donors ($10^6$ cells/mL) were stimulated with medium (Ctrl -), Alum (0.1 mg/mL), PM (50 µg/mL) or PM with alum for

18 hours. PM were adsorbed to alum with continuous stirring for 2 hours. Cell pellets were used to analyse their phenotype by flow cytometry and cell-free supernatants to quantify IL-6, IL-23, IL-12, IL-4 and IL-10 by ELISA. For inhibition experiments, hmoDCs were pre-incubated for 1 hour with 2-DG (10 mmol/L) or NAC (25 mmol/L), or for 30 minutes with rapamycin (100 mmol/L) (or corresponding vehicle controls) prior to activation. Then, cells were treated with the stimulus for 18 hours in the presence of the corresponding inhibitors to quantify IL-10 by ELISA or PD-L1 expression by flow cytometry.

Immature hmoDCs, mannan-tolDCs, Allergoid-DCs or DCs differentiated with mannan alone from nonatopic donors or allergic patients ($10^6$ cells/mL) were treated with LPS (100 ng/mL) for 18 hours at 37ºC in a humidified 5% $CO_2$ incubator. Cell pellets were used to analyse their phenotype by qPCR and cell-free supernatants to quantify IL-6, TNF-α and IL-10 by ELISA. For inhibition experiments, mannan-tolDCs were pre-incubated for 1 hour with MTA (50 µmol/L) or Pargyline (5 µmol/L) (or their vehicle control, DMSO) prior to activation. Then, cells were stimulated with LPS for 18 hours in the presence of the corresponding inhibitors to quantify TNF-α and IL-10 by ELISA.

Human MØ or Mannan-MØ were stimulated with medium (negative control) or LPS (10 ng/mL) for 18 hours. Subsequently, cells were collected and centrifuged. Cell-free supernatants were used for cytokine quantification by ELISA and pellets were used for phenotype characterization by flow cytometry or qPCR.

pDCs were cultured in complete RPMI supplemented with 10 ng/mL IL-3 at a concentration of $10^6$ cells/mL. pDCs were incubated with 5, 10 or 20 mg/mL Omalizumab for 18 hours. Cells were washed, treated with 1 μM Dexamethasone (Sigma-Aldrich), 10 μg/mL IgE-FcεR1-crosslinker or 10 μg/mL control IgG, 2 μM TLR9-L and incubated at 37ºC, 5% $CO_2$ for 18 hours. Cell-free supernatant was harvested for cytokine (IFN-α, TNF-α) analysis and cells for qPCR or flow cytometry acquisition.

For ligelizumab studies, pDCs were stripped with 1 mL of lactic acid for 5 minutes or 1 µM of DARPin bi53-79 for 1 hour. Then, cells were washed and incubated with human IgE or chimeric human IgE JW8 (IgE-NIP) for 1 hour. Cells were washed and incubated with the different stimuli.

In all cases, cell viability was analysed by trypan blue exclusion with a light microscope.

(For Articles I, II, III, IV, and Annexes I, II)

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

Previously stimulated hmoDCs or human total blood DC enriched fraction were cocultured with purified allogeneic naïve $CD4^+$ T cells (DC/T cell ratio of 1:5) for 5 days. IL-17A, IFN-γ, IL-5 and IL-10 were quantified in cell-free supernatants by ELISA. Cells were used to analyse T cell phenotype by flow cytometry using the following markers: CD3, CD4, CD127, CD25 and FOXP3.

For intracellular cytokine production, the primed $CD4^+$ T cells were washed and restimulated with 25 ng/mL PMA plus 1 mg/mL ionomycin (Sigma-Aldrich) for 6 hours. 10 µg/mL brefeldin A (BD Biosciences) was added during the last 4 hours, inhibiting cytokine secretion to extracellular medium. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Cells were stained with fluorochrome-labelled mAbs to IL-17A, IFN-γ, IL-5, and IL-10.

To analyse the induction of allergen-specific Treg cells, hmoDCs or mannan-tolDCs as well as GM-MØ or Mannan-GM-MØ from allergic patients were treated with medium or native grass pollen allergens (50 µg/mL) for 18 hours and then were cocultured with autologous peripheral blood lymphocytes (PBLs) at a DC/PBL ratio of 1:5 to analyse $FOXP3^+$ Treg cells.

pDCs were cocultured with purified allogeneic naïve $CD4^+$ T cells (1:5, pDCs: T-cell ratio) in complete RPMI supplemented with 10 ng/mL IL-3 for 5 days. For selected experiments, 5 µg/mL anti-IFNAR, 5 µg/mL anti-TNF-α, 10 ng/mL TNF-α, 5 ng/mL IFN-α, 250 µmol/L 1-MT or 0.5 µmol/L kyn were added to coculture followed by harvesting of cell-free supernatants for cytokine (IL-2, IL-5, IL-10 and IFN-γ) analysis by ELISA and cells for analysis by flow cytometry.

(For Articles I, II, III, IV, and Annexes I, II)

## 5.3. Regulatory T cell suppression assay

$CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells induced by allogeneic PM- or PM in the presence of alum-stimulated hmoDCs as well as Treg cells induced by mannan-tolDCs, were purified by cell sorting of $CD4^+CD25^{high}CD127^-$ population and mixed with CFSE-labelled autologous PBMCs (responder cells) at different ratios and stimulated with plate-bound anti-human CD3 antibody (1 mg/mL, clone OKT3; eBioscience) and soluble anti-human CD28 (1 mg/mL, clone CD28.6; eBioscience) for 5 days. For control purposes, CFSE-labelled PBMCs were cultured alone with or without stimulation, and non-Treg

cells (negative fraction of the sorting, $CD4^+CD25^-CD127^-$) were also tested. Proliferation of $CD4^+$ T cells was determined by using CFSE dilution with flow cytometry.

(For Articles I and II)

# 6. Flow cytometry

## 6.1. Surface staining

We used the same protocol for all the surface stainings. Cells were harvested, centrifuged at 300g during 5 minutes at room temperature and washed with *running buffer* [PBS/EDTA (2 mmol/L) 0.5% BSA (*bovine serum albumin*)]. Cells were suspended in 50 µL of *running buffer* and the corresponding fluorochrome-labelled antibodies were added (**Table M.4.**) and incubated for 15 minutes at room temperature in the darkness. Then, cells were washed with 1 mL of *running buffer* and centrifuged at 300g during 5 minutes at room temperature. Cell pellet was then diluted in PBS-PFA 0.1% for flow cytometry analysis in the FACScalibur (Beckton Dickinson) at CCMF from UCM.

(For Articles I, II, III, IV, and Annexes I, II)

## 6.2. Intracellular staining

For intracellular cytokine staining, T cells were first subjected to a surface staining with anti-human CD4-PerCP and CD3-APC to select $CD4^+$ T cells. Then, cells were permeabilized with Citofix/Citoperm solution (BD Biosciences) for 20 minutes in darkness at ice. After incubation time, cells were washed twice with Perm/Wash buffer (BD Biosciences) and incubated with fluorochrome-labelled monoclonal specific antibodies for cytokines (IFN-γ, IL-4, IL-17A and IL-10) for 30 minutes at room temperature in the darkness. Finally, cells were washed and diluted in 200 µL of PBS-PFA 0.1% for their analysis in FACScalibur CCMF from UCM.

(For Article I)

## 6.3. Intranuclear staining

For analysis of FOXP3 expression in human T cells primed with DCs, cells were first subjected to surface staining with anti-human CD4-PerCP, CD127-PE, and CD25-APC antibodies. Then, cells were fixed and permeabilized with FOXP3 Fix/Perm buffer for 20 minutes in darkness at room temperature. A second permeabilization was carried out with FOXP3 Perm/Wash buffer 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 described above was carried out for the phenotypic characterization of $CD4^{+}CD25^{high}FOXP3^{+}$ Treg cells in freshly isolated mouse splenocytes using anti-mouse CD4-PerCP and CD25-PE antibodies for surface staining and anti-mouse FOXP3-Alexa 488 for intranuclear staining. The corresponding isotype controls were included in each staining. Cells were diluted in 1% PBS/PFA for flow cytometry analysis in the FACScalibur at CCMF from UCM.

(For Articles I, II, III, IV, and Annexes I, II)

**Table M.4.** Flow cytometry antibodies

| Name | Supplier | Clone | Conjugate |
|---|---|---|---|
| Human FcR Blocking | Miltenyi | - | - |
| Human HLADR | Biolegend | L243 | FITC, APC |
| Human CD86 | Miltenyi | FM95 | PE |
| Human CD83 | Miltenyi | HB15 | APC |
| Human CD274 (PD-L1) | Biolegend | MIH2 | FITC |
| Human OX40-L | BD Pharmingen | Ik-1 | PE |
| Human CD127 | Biolegend | A019D5 | PE |
| Human FOXP3 | Biolegend | 259D | Alexa488 |
| Human CD4 | Biolegend | OKT4 | PerCP |
| Human CD25 | Biolegend | BC96 | APC |
| Human IL-10 | Biolegend | JES3-19F1 | PE |
| Human IL-5 | Biolegend | JES1-39D10 | PE |
| Human IL-17A | Biolegend | BL168 | Alexa488 |
| Human IFN-γ | Biolegend | 4S.B3 | Alexa488 |
| Human CD3 | BD Pharmingen | OKT3 | APC |
| Human CD19 | Biolegend | HIB19 | PE/Cy7 |
| Human CD14 | Biolegend | RMO52 | PerCP |
| Human CD1c | Biolegend | L161 | FITC |
| Human CD303 | Miltenyi | AC144 | PE |
| Human CD304 | Biolegend | 12C2 | PE/Cy7 |
| Human CD123 | Biolegend | 6H6 | FITC |
| Human IgE | Biolegend | MHE-18 | APC |
| Human CD23 | Biolegend | EBVCS-5 | PE |
| Human FcERIα | Biolegend | AER-37(CRA-1) | FITC |
| Mouse FOXP3 | Biolegend | MF-14 | Alexa488 |
| Mouse CD25 | Biolegend | PC61 | PE |
| Mouse CD4 | Biolegend | GK1.5 | PerCP |

### 6.4. CFSE staining

CFSE (carboxifluerescein diacetate N-succinimidyl ester) is a fluorescent dye that binds covalently to all free amines on the surface and inside of cells and shows little toxicity. $CD4^+$ 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 complete RPMI media was added and cells were incubated for 5 minutes on ice. Cells were washed 3 times with media and were cocultured for 5 days with hmoDCs or MØ and the corresponding stimuli.

(For Articles I and II)

## 7. Cytokine quantification by ELISA

Concentrations of IL-6, IL-10, IL-12p70, IL-1β, IL-4, IL-2, IL-5 and IFN-γ in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences). IL-23 and IL-17A levels were quantified by Human IL-23 ELISA Ready-SET-Go!® (e-Biosciences) and quantikine Elisa Kit (RD Systems), respectively. IFN-α levels were quantified with the IFN-α (pan) ELISA development kit (Mabtech). 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 1X with 5% FBS) and incubated with the standards or samples. After washing, biotinylated anti-human mAbs and streptavidin-labelled peroxidase were added to detect bound cytokines. Next, after washing, chromogenic substrate (0.63 mg/mL OPD, 0.03% $H_2O_2$ in 1 mmol/L sodium citrate) was added. The colour reaction was stopped by adding 3 N $H_2SO_4$ and the OD values were measured at 492 or 450 nm according to each kit.

(For Articles I, II, III, IV, and Annexes I, II)

## 8. Western blot

Freshly isolated hmoDCs were treated with medium (Ctrl -), PM (50 µg/mL) and PM previously adsorbed to alum (0.1 mg/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 for 15 minutes at 10,000g and 4°C, and supernatants were collected for protein quantification with Micro BCA Kit (Pierce). Ten micrograms of total protein from cell lysates was separated by means of SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane

was incubated with the indicated antibodies used as follows: phospho-PTEN (Ser380/Thr382/383) (1:1000, Cell Signaling), phospho-p70 S6 Kinase (Thr389) (1:500; Cell Signaling), phospho-Akt (Ser473) (1:1000; Cell Signaling) and β-actin (1:15000, Sigma-Aldrich) as primary antibodies and 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 multigauge software, and values were expressed relative to the β-actin loading control.

(For Article I)

## 9. RNA extraction and complementary DNA synthesis

RNA was isolated from harvested cells by using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For mRNA analysis, cDNA was generated with a PrimeScript RT Reagent Kit (Takara Bio). For miRNA analysis, RNA isolation Qiazol (Qiagen) and Total RNAzol out Mini kit (A&A Biotechnology) were used according to the manufacturer's instructions. To analyse miRNA expression, TaqMan® MicroRNA Assays (Life Technologies) and 5×HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne) were used according to the manufacturer's protocols.

(For Articles II, III, IV, and Annex I)

## 10. Real-time quantitative PCR

Real-time quantitative PCR was performed on cDNA by using FastStart Universal SYBR Green Master (Rox; Roche). The sequences of the used pair primers are shown in **Table M.5.** Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems). Data were normalized to EF1α for mRNA and let-7a for miRNAs 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 EF1α or let-7a.

(For Articles II, III, IV, and Annex I)

**Table M.5.** Primers for real-time qPCR

| 11. | Forward | Reverse |
|---|---|---|
| PDL1 | AAGATGAGGATATTTGCTGTCTTTATATTC | GTCCTTGGGAACCGTGACAGT |
| IDO | AGAAGTGGGCTTTGCTCTGC | TGGCAAGACCTTACGGACATCTC |
| SOCS1 | CCCTGGTTGTTGTAGCAGCTT | CAACCCCTGGTTTGTGCAA |
| SOCS3 | CCTCAGCATCTCTGTCGGAAGA | GCATCGTACTGGTCCAGGAACT |
| ICOSL | CTCCGCCCGCACCAT | CTACCATCGCTCTGACTTCCTTCT |

| | | |
|---|---|---|
| IL10 | GTGATGCCCCAAGCTGAGA | CACGGCCTTGCTCTTGTTTT |
| EF1α | CTGAACCATCCAGGCCAAAT | GCCGTGTGGCAATCCAAT |
| GLUT1 | GGCTTCTCCAACTGGACCTC | CCGGAAGCGATCTCATCGAA |
| HK2 | TTCGCACTGAGTTTGACCAG | TCACCAGGATAAGCCTCACC |
| GADPH | CTGCACCACCAACTGCTTAGC | TCATGTTCTGGAGAGCCCCG |
| LDHA | ATGGCAACTCTAAAGGATCAGC | CCAACCCCAACAACTGTAATCT |
| PDHA1 | ATGGAATGGGAACGTCTGTTG | CCTCTCGGACGCACAGGATA |
| ACO2 | AATGGATGTACTCGTTGGGC | ACAGCCTACTGGTGACTCGG |
| IDH3B | CTGATGCACGCCGTCAAG | GCCATATTCTGCACCTCACTCA |
| SUCLG1 | CCTACACAGCTTCTCGGCAA | CTGTGAAAGGTGCCCTGTTTG |
| SDHA | CGAACGTCTTCAGGTGCTTT | AAGAACATCGGAACTGCGAC |
| NDUFA10 | ACAGAACGCAGCAGAGTGATA | GGAAAGTGCTTGAAGCCTAGTT |
| ATP5A1 | AAGACACGCCCAGTTTCTTC | TTTGGGTTCATCTTTCATTGC |
| MNF2 | TCTGGGACCTTTGCTCATCT | CAACCAACCGGCTTTATTCC |
| HDAC1 | ACTACGACGGGGATGTTGGA | CAGCATTGGCTTTGTGAGGG |
| HDAC2 | TCTGCTACTACTACGACGGTGA | TCATTTCTTCGGCAGTGGCT |
| HDAC3 | ATCTCTGCAAGGAGCAACCC | AGCCAGAGGCCTCAAACTTC |
| HDAC4 | CCACCTCACTCCCTACCTGA | CCCAGGCCTGTGACGAG |
| HDAC5 | TCCCGTCCGTCTGTCTGTTA | GACCTGACATCCCATCCGAC |
| HDAC6 | CTGGCGGAGTGGAAGAACC | GGGGAACGGCTCCCTTTTTA |
| HDAC7 | CTCGGAGGCTGACAGTGACC | GAGGGGTCCAGGAGGAGAAT |
| HDAC8 | CAGAAGGTCAGCCAAGAGGG | GGCAGTCATAACCTAGCCCA |
| HDAC9 | GACGTGTGGTGTTGGCTCTA | GTGGCTCCAGCTCATTTCCT |
| HDAC10 | TACCATGAGGACATGACGGC | GGGATACATACTCTGGGCTGTG |
| HDAC11 | CCCGGGATGCTACACACAAC | CAGGCCCATGAAGGTGATGT |
| CCR7 | CATGGACCTGGGGAAACCAA | AAAGTTCCGCACGTCCTTCT |
| CD80 | GATAACCTGCTCCCATCCTGG | CTTGGGGCAAAGCAGTAGGT |
| CD206 | ACACAAACTGGGGGAAAGGTT | TCAAGGAAGGGTCGGATCG |
| CD163 | AAATTACCTGCTCAGCCCACA | ACGTGTCACCATGCTTCACT |
| CCL2 | TCTGTGCCTGCTGCTCATAG | GGGCATTGATTGCATCTGGC |
| IL6 | GGTACATCCTCGACGGCATCT | GTGCCTCTTTGCTGCTTTCAC |
| IL10 ChIP | TTGCCTGGGAAGTGGGTGCA | AGAAGGCATGCACAGCTCAGC |
| TNF ChIP | ACCACGCTCTTCTGCCTGCT | TCCATCCCTCCCTATCAGCGCA |
| PDL1 ChIP | GCTTCCGCCGATTTCACCGA | TCGGGAAGCTGCGCAGAACT |
| IDO ChIP | AGGTTGTGTTTCCGGGCTGCT | ACAGGTGGCCGGAGAAGAACA |
| SOCS1 ChIP | TCGGCCCTGTTTCCCTCTCT | CCTGGCGGCAGAAAGTGGAA |
| SOCS3 ChIP | GGTCCCGAATCGAAGTCTCCGT | ACTTCACGGCCGCCAACATCT |
| B2M ChIP | CTCTCTCGCTCCGTGACT | GCCGAAAGGGGCAAGTAG |
| MYOD1 ChIP | GTAGGAGAGGCGGGAGAA | TAGGTCTGCGAGGGTCTC |

## 11. Metabolic studies

The Warburg effect in stimulated-hmoDC cultures was determined photometrically 18 hours after stimulation by quantifying the OD at 570 nm and calculating the Warburg effect as 1/OD570 normalized to the unstimulated. Glucose concentrations in 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 DCs (glucose concentration in RPMI 1640 = 2 mg/mL). Lactate concentrations in culture supernatants were determined 18 hours after stimulation by using the colorimetric L-Lactate Assay kit (Abcam), according to the manufacturer's recommendations. To measure mitochondrial mass and mitochondrial membrane potential, MitoTracker Green FM (25 nmol/L) or MitoTracker Red CMXRos (250 nmol/L, Thermo Fisher Scientific) were used. Arbitrary fluorescence values were obtained in a FLUOstar Omega microplate reader (Ex/Em = 490/516 nm for MitoTracker Green and Ex/Em = 579/599 nm for MitoTracker Red CMXRos). Total $NAD^+$/nicotinamide adenine dinucleotide (NADH) content, $NAD^+$, and NADH concentrations in lysates of DCs were determined by using NAD/NADH Assay Kit (Abcam), according to the manufacturers' recommendations. Reactive oxygen species (ROS) were measured by DCFDA Cellular ROS Detection Assay Kit (Abcam) following manufacturer's instructions. Briefly, total ROS levels were measured using the cell-permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA). Cells were stained with DCDFA for 30 minutes at 37°C after 18 hours of treatment with the corresponding stimuli. Fluorescence was measured in a FLUOstar Omega microplate reader (Ex/Em = 485/535 nm). ATP concentration in lysates of hmoDCs were determined with the ATP Determination Kit (Invitrogen) according to the manufacturer's instructions.

(For Articles I and II)

## 12. Transfection experiments

Transfection with NickFect71 was performed as previously described.[188] Briefly, NickFect71 was incubated with miRNA mimic or lock nucleic acid (LNA) as inhibitor at a 17:1 CPP (cell-penetrating peptide):miRNA molar ratio in MQ-water in 1/10 of final treatment volume at room temperature for 1 hour to form CPP:miRNA complexes; mixed with media and added to the cells. After 18 hours, mannan-tolDCs were stimulated with 100 ng/mL of LPS for 18 hours or left unstimulated. Transfections in DCs were performed at 100 nmol/L of miRNA mimic negative control, miR-155-5p (from Life Technologies)

and miRCURY LNA™ inhibitors (microRNA Power inhibitor hsa-miR-146 and Negative control A, Exiqon). Cells were harvested in Qiazol and kept at -80ºC until RNA isolation.

(For Article II)

## 13. Chromatin immunoprecipitation (ChIP)

HmoDCs and mannan-tolDCs were fixed after differentiation. Cells were subjected to lysis and sonication using the Bioruptor UCD-200 sonicator (Diagenode) to obtain chromatin fragments of 200–400 bp. Sheared chromatin was immunoprecipitated with 2 µL of anti-H3K4me3 antibody (Millipore, 07-473), 2 µL of anti-H3K27me3 antibody (Millipore, 07-449), 2 µL of anti-H3K27ac antibody (Abcam, ab4729) or 2 µL of anti-IgG antibody (Millipore, 12-370) and 3 µL of Dynabeads M-280 magnetic beads (Sheep Anti-Mouse IgG, Sheep Anti-Rabbit IgG, Life Technologies) using the SX-8G IP-Star Automated System (Diagenode). ChIP samples were decrosslinked at 65°C for 4 hours. After incubation, the samples were treated with 0.2 mg/mL RNaseA (RNase Cocktail Enzyme Mix, Ambion, Life Technologies) and 0.4 mg/mL Proteinase K (Thermo Scientific). DNA was purified with the DNA Clean kit & Concentrator TM-5 (Zymo Research) according to the manufacturer's protocol. Real-time quantitative PCR was performed on the purified ChIP and INPUT DNAs by using FastStart Universal SYBR Green Master. Sequences of the used pair primers are in **Table M.5**. Data from H3K4me3 and H3K27ac marks were normalized to B2M positive control and data from H3K27me3 mark were normalized to MYOD1 negative control.

(For Article II)

## 14. Immunization of mice with control, alum, PM, or PM with alum: quantification of induced serum allergen-specific immunoglobulins levels; and cytokine profiles of splenocytes stimulated *in vitro* with native grass pollen allergens

BALB/c female mice (6 weeks old) were immunized 3 times with 20 µg (200 µL at 100 µg/mL) of PM, or PM previously adsorbed to 2 mg of alum by means of subcutaneous injection every 15 days and killed 7 days after the last immunization. Serum specific IgG2a, IgG1, IgE reactivities to native grass pollen allergens induced with each treatment were determined by means of ELISA. Spleens were used to prepare single-cell suspensions according to conventional protocols. The presence of splenic $CD4^{+}CD25^{high}FOXP3^{+}$ Treg cells in the different immunized mice was quantified, as

described above. Splenocytes were stimulated *in vitro* with N, and cytokine (IFN-γ, IL-5, IL-4 and IL-10) production was measured by using CBA Cytometric kits (Beckton Dickinson) in 48-hour culture supernatants. Animal experiments were approved by the Ethics Committee of Hospital Clínico San Carlos.

(For Article I)

## 15. ELISA inhibition of the binding of serum IgE from allergic patients to N extract by blocking antibodies induced after immunization of mice

The blocking capacity of the serum antibodies generated by means of immunization of mice with control, alum, PM, and PM with alum was analysed by using ELISA inhibition assays. Microtiter plates (Corning Inc) were coated with 10 µg of N in 100 µL of PBS overnight at 4°C. Plates were washed 3 times with PBS and 0.1% vol/vol Tween-20 and blocked for 1 hour with PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. Then plates were incubated with the serum of mice immunized against negative control, alum, PM, or PM with alum extracts. After 1 hour of incubation, plates were washed 4 times with PBS and 0.1% vol/vol Tween-20 and incubated for 2 hours with individual serum from 5 patients with well-defined grass pollen allergy diluted 1:50 in PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. The patients were previously described and the study was approved by the Regional Ethics Committee of Comunidad Autónoma de Madrid (EudraCT: 2014-005471-88).[60] After 4 washes, bound IgE antibodies were detected by incubating for 1 hour with biotin anti-human IgE rabbit monoclonal antibody (1:100000 diluted) (RevMab Biosciences), followed by 45 minutes with horseradish peroxidase–coupled streptavidin (diluted 1:2500). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% $H_2O_2$ and 0.63 mg/mL *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0), and the reaction was stopped with 3 N $H_2SO_4$. OD was measured at 492 nm in an ELISA reader.

(For Article I)

## 16. ELISA of serum-specific IgE from allergic patients to native grass pollen allergens

Microtiter plates (Corning Inc) were coated with 10 µg of N in 100 µL of PBS overnight at 4°C. Plates were washed 3 times with PBS and 0.1% vol/vol Tween-20 and blocked for 1 hour with PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. Then plates were incubated for 2 hours with individual serum from patients with well-defined grass

pollen allergy diluted in PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. After 4 washes, bound IgE antibodies were detected by incubating for 2 hours with goat anti-human IgE-biotin (1:2000 diluted) (ThermoFisher), followed by 1 hour with horseradish peroxidase–coupled streptavidin (diluted 1:500). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% $H_2O_2$ and 0.63 mg/mL *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0), and the reaction was stopped with 3 N $H_2SO_4$. OD was measured at 492 nm in an ELISA reader.

(For Articles II, III, IV, and Annexes I, II)

## 17. Statistical analysis

In all experiments, data represent the mean ± SEM of the indicated parameters. For DCs experiments, statistical differences were determined with the paired or unpaired Student *t* test when data follow normal distribution and Wilcoxon matched-pairs test when they not. For MØ experiments, statistical analysis was calculated using one-way ANOVA, whose P value summary was statistically significant ($P < 0.05$) in all the cases, or Wilcoxon test/ Mann-Whitney test when comparing just two different conditions. For pDCs experiments, correlation analysis was performed by Spearman´s correlation test.

In all the cases, Prism software 6.0 (GraphPad Software) has been used and significance is indicated in each figure. Significance was defined as $*P< 0.05$, $**P<0.01$, and $***P<0.001$.

(For Articles I, II, III, IV, and Annexes I, II)

# BLOCK A: Study of the molecular mechanisms involved in the mode of action of next-generation vaccines for AIT targeting DCs

# ARTICLE I: Alum impairs tolerogenic properties induced by allergoid-mannan conjugates inhibiting mTOR and metabolic reprogramming in human DCs

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Polymerized allergoids conjugated to nonoxidized mannan (PM) represent novel suitable AIT vaccines targeting DCs. Alum is the most used adjuvant in AIT, but the detailed molecular mechanisms underlying its adjuvanticity remained unknown. To assess the impact of the presence of alum in the imprinted effects of PM, hmoDCs and an enriched fraction of total DCs were treated with PM alone or with PM previously adsorbed to alum. Alum decreased PD-L1 expression and IL-10 production and increased the production of pro-inflammatory cytokines induced by PM in human DCs. The influence of alum in T cell polarization was studied by coculture experiments of DCs and allogeneic $CD4^+$ naïve T cells. Alum impaired the generation of Treg cells while inducing Th1/Th2/Th17 polarization. The inhibition of the tolerogenic properties imprinted by PM were corroborated in mice. Regarding the molecular mechanisms implicated in the action of alum, mTOR activation was abolished and alum induced metabolic reprogramming of DCs with a shift in the glycolytic pathway and induction of ROS production in PM-activated DCs. These results provide novel insights into the way of action of allergoid-mannan conjugates and alum, and might well contribute to improve the formulation of other novel vaccines for AIT.

*Herein, this study is presented with the signed consent of all the co-authors. During the research, I performed all the human experiments and participated in the general experimental design, in the analysis and discussion of the data, and also in the organization, writing and revision of the manuscript.*

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ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

# Alum impairs tolerogenic properties induced by allergoid-mannan conjugates inhibiting mTOR and metabolic reprogramming in human DCs

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## Abstract

**Background:** Polymerized allergoids conjugated to mannan (PM) are suitable vaccines for allergen-specific immunotherapy (AIT). Alum remains the most widely used adjuvant in AIT, but its way of action is not completely elucidated. The better understanding of the mechanisms underlying alum adjuvanticity could help to improve AIT vaccine formulations.

**Objective:** We sought to investigate the potential influence of alum in the tolerogenic properties imprinted by PM at the molecular level.

**Methods:** Flow cytometry, ELISAs, cocultures, intracellular staining and suppression assays were performed to assess alum and PM effects in human dendritic cells (DCs). BALB/c mice were immunized with PM alone or adsorbed to alum. Allergen-specific antibodies, splenocyte cytokine production and splenic forkhead box P3 (FOXP3)$^+$ regulatory T (Treg) cells were quantified. Metabolic and immune pathways were also studied in human DCs.

**Results:** Alum decreases PD-L1 expression and IL-10 production induced by PM in human DCs and increases pro-inflammatory cytokine production. Alum impairs PM-induced functional FOXP3$^+$ Treg cells and promotes Th1/Th2/Th17 responses. Subcutaneous immunization of mice with PM plus alum inhibits in vivo induction of Treg cells promoted by PM without altering the capacity to induce functional allergen-specific blocking antibodies. Alum inhibits mTOR activation and alters metabolic reprogramming by shifting glycolytic pathways and inhibiting reactive oxygen species (ROS) production in PM-activated DCs, impairing their capacity to generate functional Treg cells.

**Conclusion:** We uncover novel mechanisms by which alum impairs the tolerogenic properties induced by PM, which might well contribute to improve the formulation of novel vaccines for AIT.

**Abbreviations:** 2-DG, 2-deoxy-D-glucose; AIT, allergen-specific immunotherapy; Alum, aluminium hydroxide; Breg, regulatory B; CLRs, C-type lectin receptors; DCs, dendritic cells; FOXP3, forkhead box P3; HmoDC, human monocyte-derived dendritic cell; mDC, myeloid dendritic cell; mTOR, mammalian target of rapamycin; N, native grass pollen extract; NAC, N-acetyl-L-Cysteine; OX40L, OX40 ligand; pDC, plasmacytoid dendritic cell; PD-L1, programmed death-ligand 1; PM, polymerized allergoids conjugated to mannan; PTEN, phosphatase and tensin homologue; ROS, reactive oxygen species; Treg, regulatory T; $\Delta\psi$, membrane potential.

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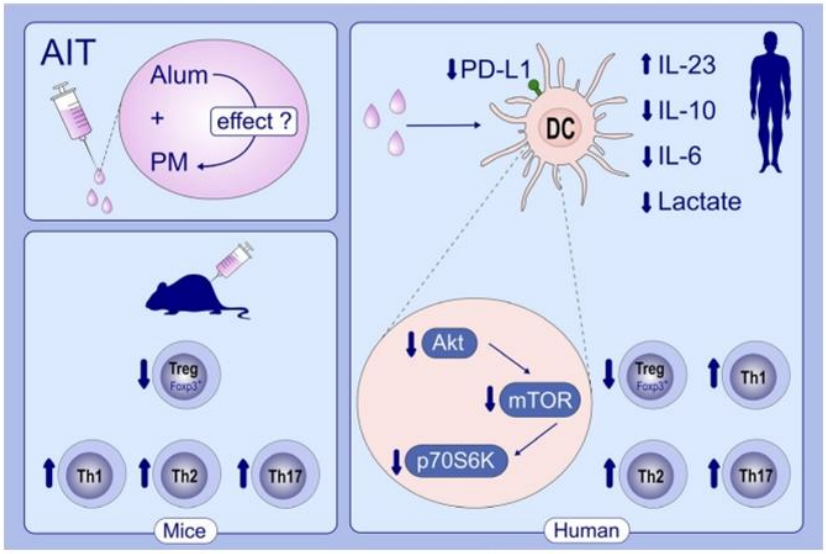
KEYWORDS
allergen-specific immunotherapy, alum, dendritic cells, polymerized allergoids conjugated to mannan, regulatory T cells




**GRAPHICAL ABSTRACT**
This study investigated the potential influence of alum in the tolerogenic properties imprinted by PM. Alum decreased expression of PD-L1 and production of IL-10, IL-6 and lactate, increased IL-23 release and inhibited mTOR activation in PM-activated DCs. Alum suppressed PM-induced functional FOXP3$^+$ Treg cells and promoted Th1/Th2/Th17 responses.

## 1 | INTRODUCTION

Allergen-specific immunotherapy (AIT) is the only treatment with potential long-lasting disease-modifying effects for allergic diseases.[1-3] Successful AIT is associated with a very rapid desensitization of mast cells and basophils and with the inhibition of type 2 immune-mediated responses. Sustained tolerance after AIT discontinuation requires the generation and preservation of functional allergen-specific regulatory T (Treg) and B (Breg) cells as well as allergen-specific blocking antibodies.[4-6] Clinical trials and real-life practice demonstrated AIT as an effective treatment; however, it still faces several drawbacks regarding the long treatment duration, patient compliance, side effects or low efficacy for some patients.[3,7,8] Polymerized allergoids conjugated to mannan (PM) have been recently reported as next-generation vaccines targeting dendritic cells (DCs) that might well contribute to overcome such inconveniences.[9-12] PM are captured very efficiently by human DCs via mannose receptor and other C-type lectin receptors (CLRs). PM activates CLRs and promotes IL-10-producing tolerogenic DCs that generate functional forkhead box P3 (FOXP3)$^+$ Treg cells through programmed death-ligand 1 (PD-L1).[9,10] In mice, subcutaneous or sublingual immunization with PM increases the frequency of splenic FOXP3$^+$ Treg cells and induces healthy immune responses to allergens.[9,11,13] All these beneficial effects depend on the structural integrity of the conjugated mannan.[9,12-14]

Aluminium hydroxide (alum) remains the most widely used adjuvant in AIT.[15-17] For long time, the main effects attributed to alum have been related to its depot effect leading to the slow release of the adsorbed allergens.[15,18] Alum also activates innate immune responses in DCs and macrophages. Alum induces the release of uric acid and double-stranded DNA, which activate NLRP3 inflammasome and IRF3 leading to the production of pro-inflammatory cytokines.[19,20] In addition, alum activates Syk-PI3kδ pathways and induces PGE2 production, which is involved in Th2 polarization.[19,20] However, the detailed molecular mechanisms driving alum adjuvanticity still remain largely unknown.[15,18]

Metabolic reprogramming in immune cells refers to the changes occurring after activation in the intracellular metabolic pathways that lead to functional regulation.[21] DCs' function is finely regulated by metabolic reprogramming in cooperation with immune pathways.[22] The change in metabolism towards glycolysis with high lactate production despite the availability of oxygen is known as the Warburg effect, which represents a key event in the regulation of DCs' function.[21,22] Mammalian target of rapamycin (mTOR) is an important regulator of metabolic reprogramming, and its activation state is highly responsive to intracellular and extracellular signals.[21,22] How DCs simultaneously activated with alum and CLR ligands drive immune responses and the potential consequences of this cross-talk in metabolic reprogramming remains fully elusive.

The better understanding of the mechanisms involved in alum adjuvanticity might well contribute to improve vaccine formulations for AIT. Herein, we show for the first time that alum impairs the tolerogenic features imprinted by allergoids conjugated to mannan in human DCs. Alum inhibits PM-induced functional FOXP3$^+$ Treg cells and promotes Th1/Th2/Th17 responses. In vivo, subcutaneous immunization of mice with PM adsorbed to alum inhibits the healthy immune responses to allergens induced by PM alone. Alum shifts glycolytic pathways, inhibits mTOR activation and impairs reactive oxygen species (ROS) production in PM-activated DCs, thus inhibiting their capacity to generate functional Treg cells. Collectively, we uncover novel mechanisms by which alum impairs the tolerogenic properties induced by PM, which might well pave the way not only for the future rational design of novel AIT vaccines but also for other vaccine formulations.

## 2 | METHODS

### 2.1 | Material, media and reagents

For cell cultures, we used RPMI 1640 (Lonza) supplemented with 10% heat-inactivated foetal bovine serum, 100 µg/mL normocin (InvivoGen), 50 µg/mL penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mmol/L sodium pyruvate (Life Technologies). Glutaraldehyde-polymerized grass pollen (*Phleum pratense)* allergoids conjugated to nonoxidized mannan (PM) and native grass pollen *P pratense* allergens (N) were provided by Inmunotek SL. Aluminium hydroxide gel (Alhydrogel) was from InvivoGen. Inhibitors for mTOR (rapamycin) (InvivoGen), ROS (N-acetyl-cysteine (NAC)) or glycolysis (2-Deoxy-D-glucose (2-DG)) (Sigma-Aldrich) were used for the inhibition experiments.

### 2.2 | Cell cultures

Immature hmoDCs or human total blood DCs from healthy donors or allergic patients ($10^6$ cells per mL) were stimulated with medium (Ctrl -), alum (0.1 mg/mL), PM (50 µg/mL) or PM with alum for 18 hours. PM were adsorbed to alum with continuous stirring for 2 hours. Cell pellets were used to analyse their phenotype by flow cytometry and cell-free supernatants to quantify IL-6, IL-23, IL-12, IL-4 and IL-10 by ELISA. For inhibition experiments, hmoDCs were preincubated for 1 hour with 2-DG (10 mmo/L) or NAC (25 mmo/L), or for 30 minutes with rapamycin (100 nmo/L) (or corresponding vehicle controls) prior to activation. Then, the cells were stimulated with the stimulus for 18 hours in the presence of the corresponding inhibitors to quantify IL-10 by ELISA or PD-L1 by flow cytometry. Cell viability was analysed in all the cases by trypan blue exclusion with a light microscope.

### 2.3 | Statistical analysis

In all experiments, data represent the mean ± SEM of the indicated parameters. Statistical differences were determined with the paired or unpaired Student *t* test using Prism software 6.0 (GraphPad Software). Significance is indicated in each figure.

All procedures used in this study are fully described in the Methods section in this article's Data S1.

## 3 | RESULTS

### 3.1 | Alum impairs tolerogenic features imprinted by PM in human DCs

To analyse the impact of alum on the expression pattern of different surface molecules and cytokine production induced by PM in human DCs, we treated human monocyte-derived dendritic cells (hmoDCs) or an enriched fraction of total DCs with PM alone or with PM plus alum. The expression of the inhibitory molecule PD-L1 was significantly reduced in PM-stimulated hmoDCs in the presence of alum (Figure 1A). In contrast, alum significantly increased the expression of CD-83 and OX40 ligand (OX40-L), which are molecules associated with mature DCs and amplification of Th2 cell responses, respectively.[23,24] There were no significant differences in HLA-DR expression (Figure 1A). Representative histograms are displayed in Figure 1B. HmoDCs activated by PM in the presence of alum produced significantly higher levels of the pro-inflammatory cytokine IL-23 than PM-stimulated hmoDCs (Figure 1C). We did not detect IL-12 or significant differences in IL-4 production (Figure 1C). Alum significantly reduced the production of IL-6 and the anti-inflammatory cytokine IL-10 in PM-activated hmoDCs (Figure 1C). Remarkably, alum alone only induced significant production of IL-23 but not any of the other assayed cytokines. Next, we isolated an enriched fraction of human blood total DCs containing both plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) (Figure 1D). Supporting our data in hmoDCs, alum significantly increases the production of IL-23 by PM-treated total blood DCs. IL-10 and IL-6 production also tends to decrease in the presence of alum in PM-activated total blood DCs (Figure 1E).

### 3.2 | Alum promotes Th1, Th2 and Th17 responses and decreases IL-10-producing T cells induction by PM-activated human DCs

To determine the capacity of human DCs stimulated with PM in the presence of alum to polarize CD4$^+$ T-cell responses, we performed coculture experiments. HmoDCs activated with PM plus alum generated T cells producing higher levels of IFN-γ, IL-5 and IL-17A than hmoDCs stimulated with PM alone (Figure 2A). In contrast, PM-activated hmoDCs in the presence of alum generated T cells producing less IL-10 than PM-activated hmoDCs (Figure 2A). The IFN-γ/IL-10, IL-5/IL-10 and IL-17A/IL-10 ratios were significantly higher when T cells were primed by hmoDCs activated with PM plus alum than with PM alone (Figure 2B). To verify these data at the single-cell level, we performed intracellular staining experiments. Primed CD4$^+$ T cells were restimulated with PMA/ionomycin in the presence of brefeldin A. Supporting our results, the percentages of IFN-γ-, IL-5- and IL-17A-producing CD4$^+$ T cells generated by hmoDCs activated with PM in the presence of alum were significantly higher than

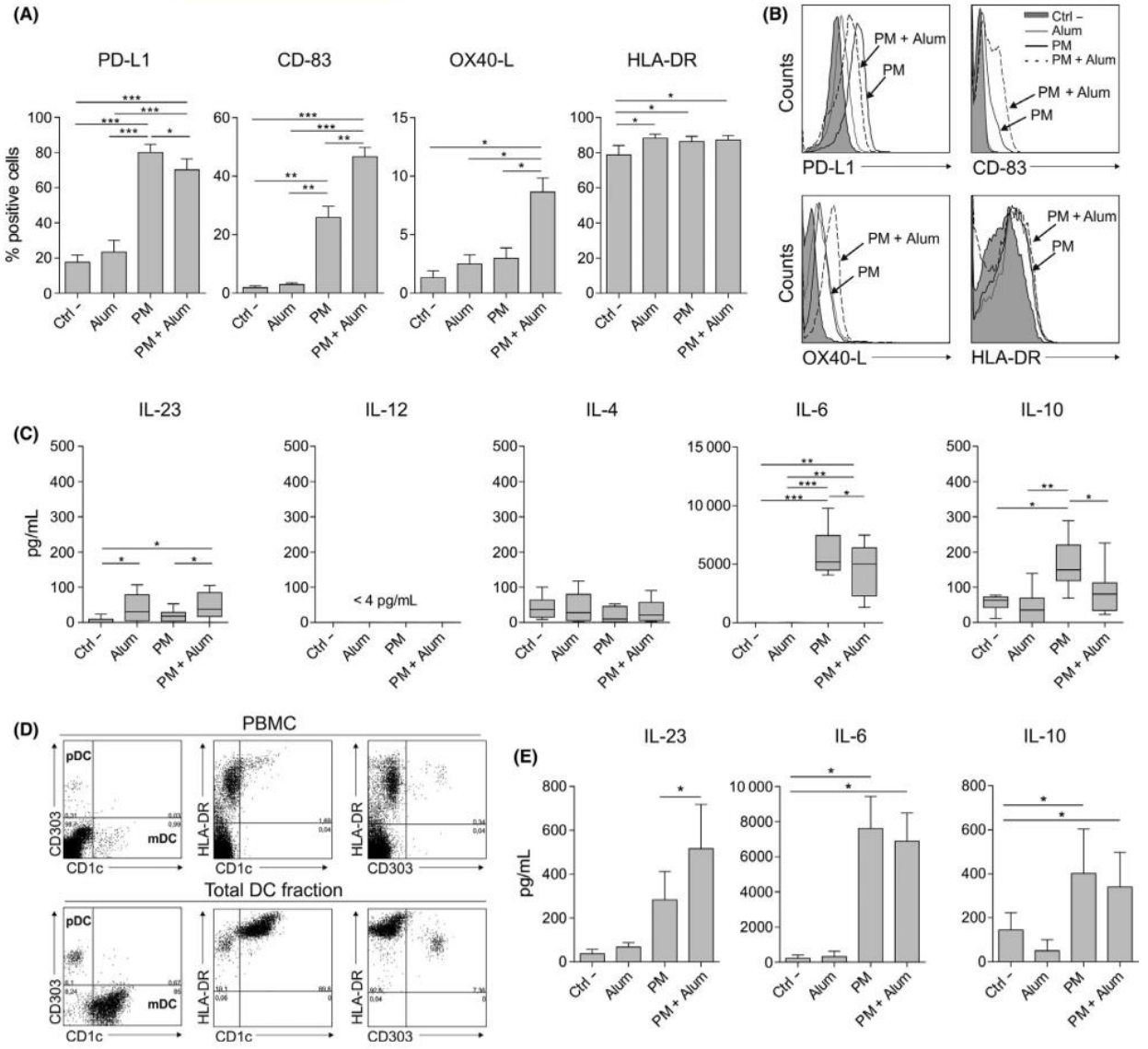



**FIGURE 1** Alum alters the phenotype and function induced by PM in human DCs from healthy donors. A, Percentage of positive cells after stimulation of hmoDCs with medium (Ctrl -), alum, PM or PM with alum for 18 h (n = 5-7). B, Flow cytometry representative histograms. C, Cytokine production after stimulation of hmoDCs with the indicated stimulus for 18 h (n = 7). D, Representative dot plots for pDCs and mDCs in PBMCs and the enriched total DC fraction. E, Cytokine production after stimulation of total blood DCs with the indicated stimulus for 18 h (n = 6). Paired Student *t* test: **P* < .05, ***P* < .01 and ****P* < .001

with PM alone. The percentage of IL-10-producing $CD4^+$ T cells was significantly decreased in the presence of alum (Figure 2C). Representative dot plots are displayed in Figure 2D. Coculture experiments using an enriched fraction of total blood DCs demonstrated that T cells primed by blood DCs activated with PM in the presence of alum produced higher amounts of IFN-γ, IL-5 and IL-17A and significantly lower levels of IL-10 than T cells generated by total DCs activated with PM alone (Figure 2E). The IFN-γ/IL-10, IL-5/IL-10 and IL-17A/IL-10 ratios were significantly increased in the presence of alum (Figure 2F). Similar results were obtained in longer cocultures performed during 10 days (data not shown).

### 3.3 | Alum impairs the generation of functional $FOXP3^+$ Treg cells by PM-activated DCs

PM-treated hmoDCs induced significantly higher numbers of $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells than medium- or alum-treated hmoDCs. In the presence of alum, the number of $FOXP3^+$ Treg cells induced by PM-activated hmoDCs was significantly decreased (Figure 3A). For functional experiments, we sorted the generated Treg cells ($CD4^+CD25^{high}CD127^-$) (Figure S1A). Purified Treg cells generated by PM-activated hmoDCs suppressed autologous PBMCs in a dose-dependent manner (Figure S1B) and displayed a

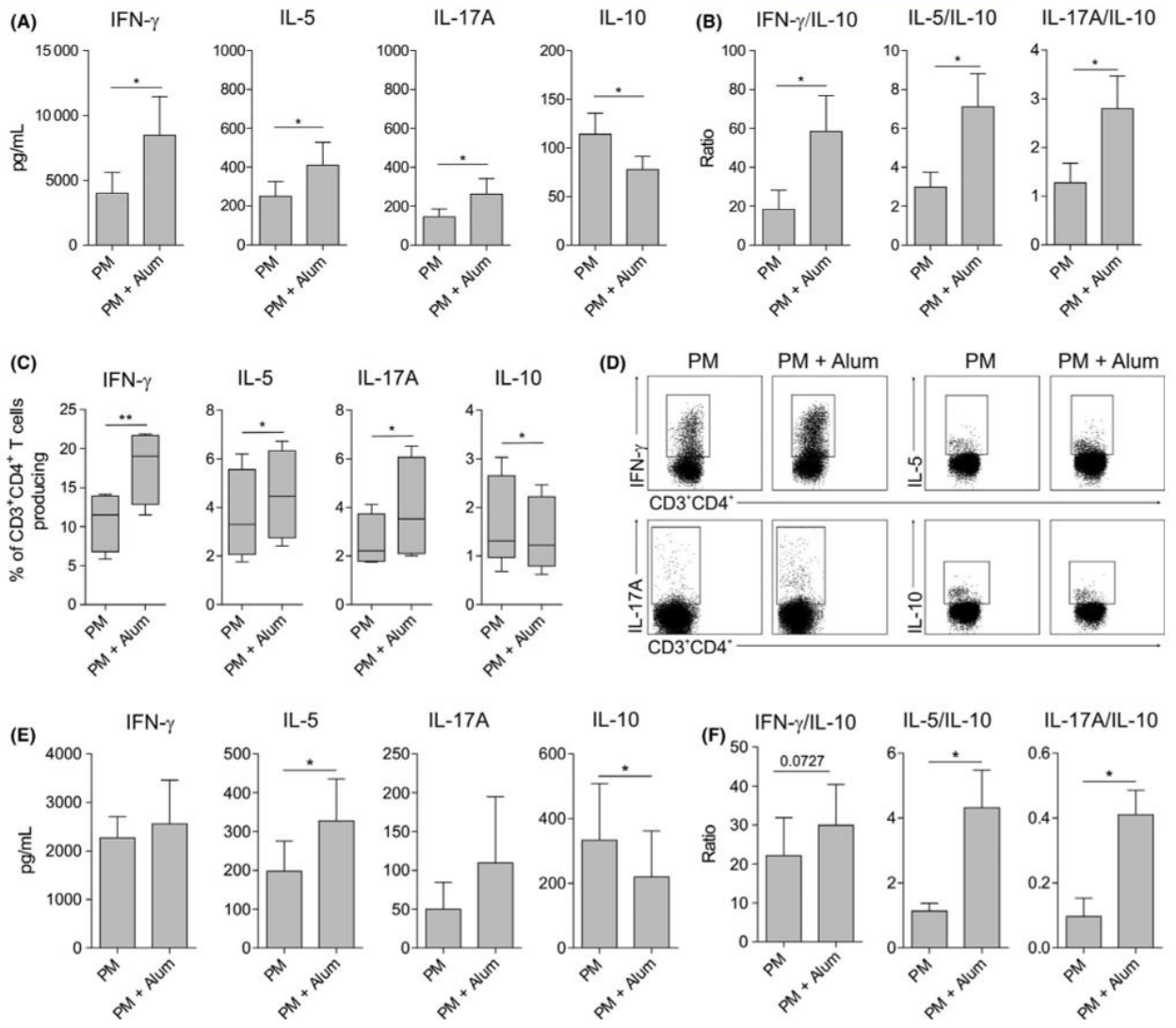



**FIGURE 2** Alum promotes the generation of Th1, Th2 and Th17 cells and impairs the induction of IL-10-producing T cells by PM. A and B, Cytokines (A) or cytokine ratios (B) produced by allogeneic naïve $CD4^+$ T cells primed by PM- or PM and alum-activated hmoDCs from healthy donors after 5 d (n = 6). C, After 5 d of coculture, primed T cells were washed and stimulated for 6 h with PMA/ionomycin in the presence of brefeldin A. Percentage of $CD3^+CD4^+$ T cells producing the indicated cytokines after intracellular staining and flow cytometry analysis (n = 4). D, Representative dot plots for the intracellular staining after flow cytometry analysis. E and F, Cytokines (E) or cytokine ratios (F) produced by allogeneic naïve $CD4^+$ T cells primed by PM- or PM and alum-activated total blood DCs from healthy donors after 5 d (n = 5). Paired Student *t* test: $^*P < .05$ and $^{**}P < .01$

more potent suppression capacity than those generated in the presence of alum (Figure 3B). Purified non-Treg cells ($CD4^+CD25^-CD127^-$) from both conditions showed no suppression capacity (Figure 3B). Interestingly, PM-activated total DCs also generated a large number of $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells, which was significantly reduced in the presence of alum, thus confirming the results obtained with hmoDCs (Figure 3C). We obtained the same results when hmoDCs from allergic patients were employed (Figure S2A). The cytokine signature of PBMCs from allergic patients and healthy donors stimulated with PM alone or with PM in the presence of alum was also comparable and characterized by reduced IL-10 production and increased INF-γ/IL-10 ratio in the presence of alum (Figure S2B,C). These results confirm our previous data showing that PM is able to induce similar responses in allergic patients and healthy donors.[9]

### 3.4 | Immunization of BALB/c mice with PM in the presence of alum impairs the healthy immune responses to allergens imprinted by PM

To assess the in vivo relevance of our findings, we immunized subcutaneously BALB/c mice with diluent (Ctrl -), alum, PM or PM plus alum, following the protocol showed in Figure 4A and analysed the

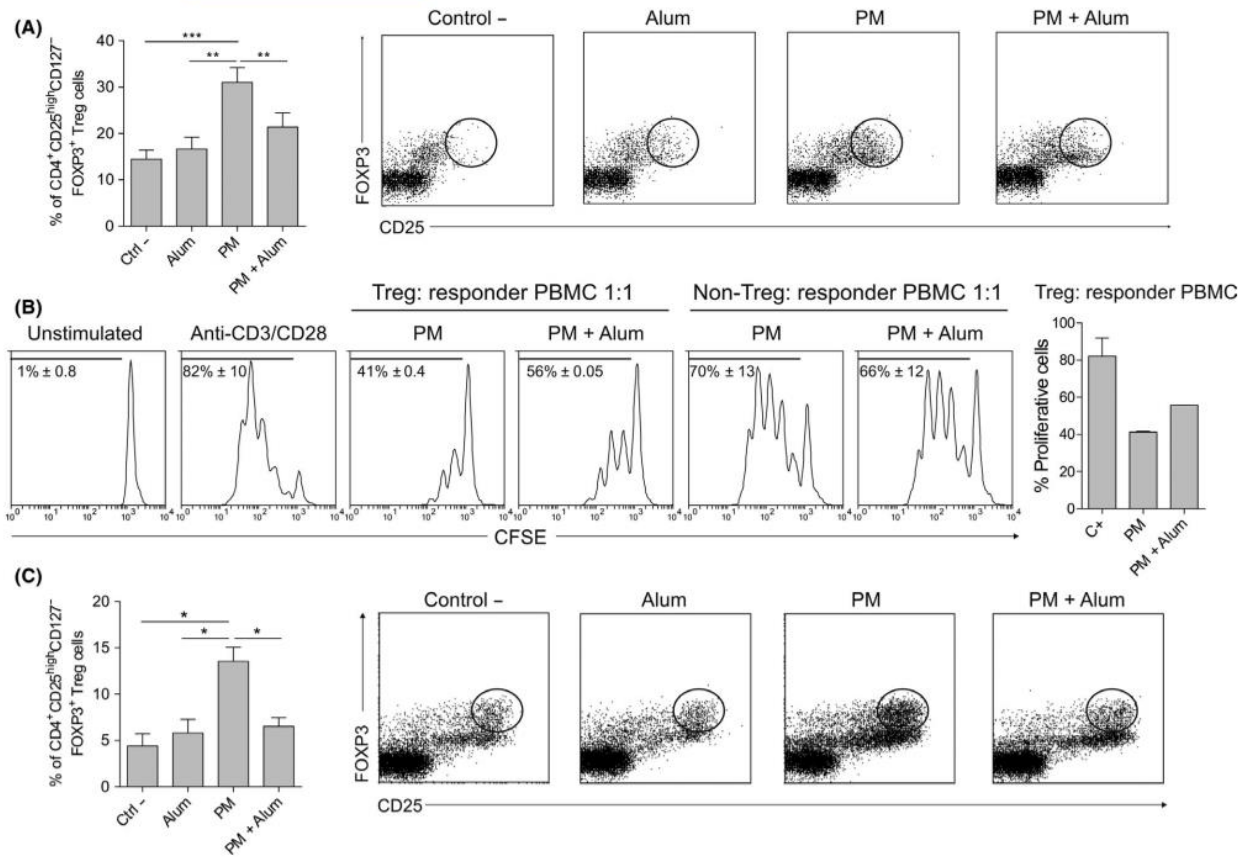



**FIGURE 3** Alum reduces PM-induced functional FOXP3$^+$ Treg cells. A, Percentage of induced CD4$^+$CD25$^{high}$CD127$^-$FOXP3$^+$ Treg cells by allogeneic medium (Ctrl -)-, alum-, PM- or PM and alum-treated hmoDCs from healthy donors after 5 d (gating in lymphocytes, n = 9). B, Proliferation of CFSE-labelled PBMCs gated on CD4$^+$ T cells after 5 d of coculture with autologous purified FOXP3$^+$ Treg cells generated by allogeneic PM- or PM and alum-treated hmoDCs (ratio 1:1). The percentage of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition at a 1:1 ratio is shown (n = 2). C, CD4$^+$CD25$^{high}$CD127$^-$FOXP3$^+$ Treg cells generated by allogeneic medium (Ctrl -)-, alum-, PM- or PM and alum-treated total blood DCs after 5 d (gating in lymphocytes, n = 4). Paired Student *t* test: $^*P < .05$, $^{**}P < .01$ and $^{***}P < .001$

indicated parameters. Splenocytes from mice immunized with PM plus alum produced significantly higher levels of IFN-γ, IL-5 and IL-4 but less IL-10 after in vitro stimulation with native grass pollen extract (N) than those from mice immunized with PM alone (Figure 4B). The ratios IL-10/IFN-γ and IL-10/IL-5 were significantly lower in the presence of alum (Figure 4C). Interestingly, the percentage of splenic FOXP3$^+$ Treg cells was significantly higher in mice immunized with PM than diluent (Ctrl -), which was significantly impaired in the presence of alum (Figure 4D). The levels of serum IgG1 and IgE antibodies specific for native grass pollen allergens were higher in mice immunized with PM plus alum than in mice immunized only with PM, whereas the levels of IgG2a were not significantly different between these two groups (Figure 4E). The IgG1/IgG2a ratio was significantly higher in mice immunized with PM plus alum than with PM alone (Figure 4F). Next, we assessed the capacity of the antibodies generated in mice after each immunization to block patients' IgE binding to native grass pollen extract. Although the antibodies induced with PM in the presence of alum display a slight higher blocking activity than those generated by PM alone, significant differences were not observed, indicating that alum does not significantly enhance the capacity of PM to generate allergen-specific blocking antibodies.

### 3.5 | Alum alters metabolic reprogramming induced by PM and inhibits mTOR activation in human DCs

We studied changes in the metabolic state of DCs activated with PM and how this could be influenced by the presence of alum. HmoDCs activated with PM increased the production of lactate (Warburg effect) compared with unstimulated hmoDCs, which was significantly reduced when hmoDCs were stimulated with PM plus alum (Figure 5A,B). The stimulation of hmoDCs with PM significantly increased the consumption of glucose from the culture medium (Figure 5C), thus significantly enhancing the metabolic activity (Figure 5D). We did not detect significant differences in glucose consumption or metabolic rates when DCs were stimulated with PM in the presence of alum (Figure 5C,D),

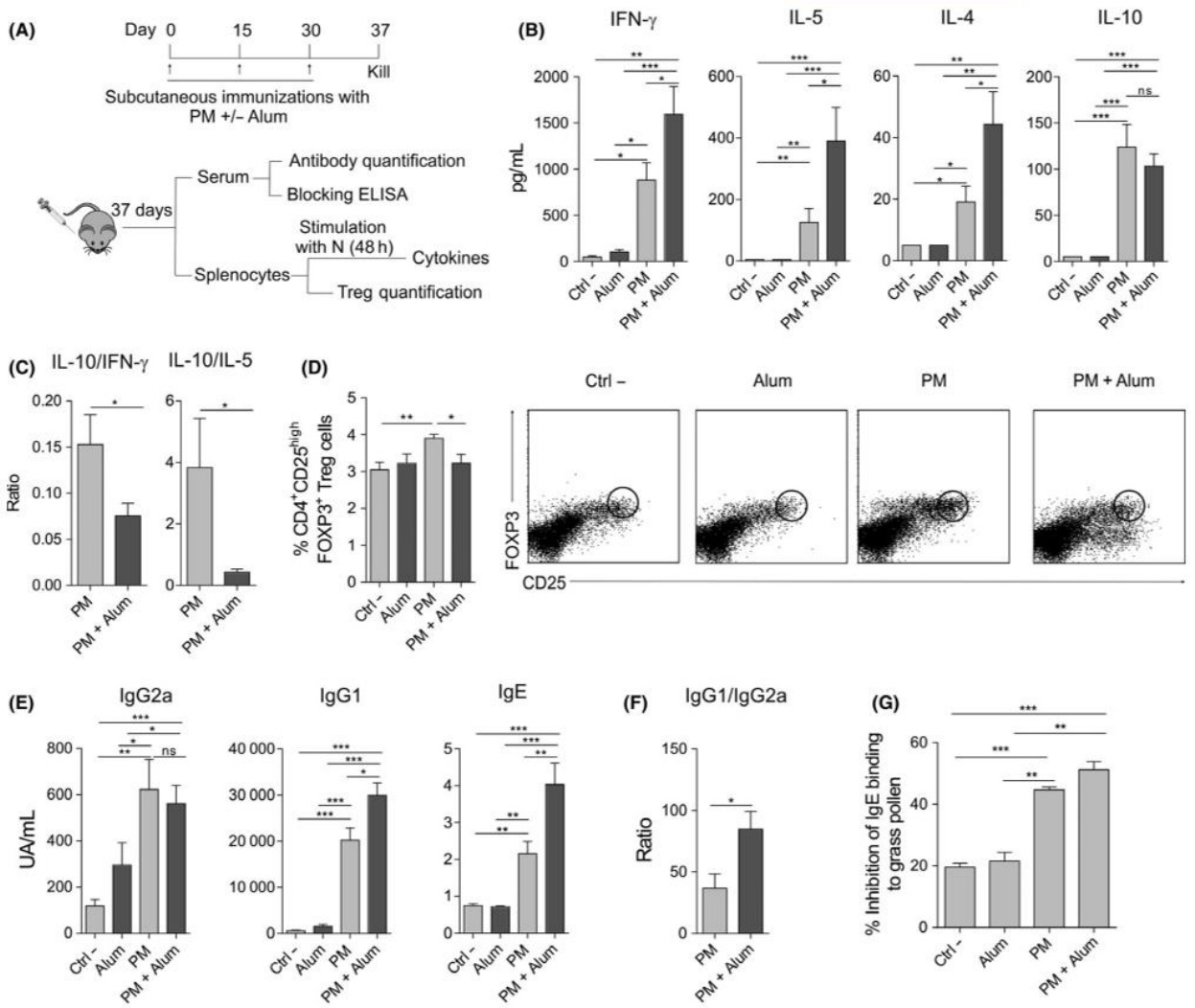



**FIGURE 4** Alum impairs in vivo tolerogenic properties induced by PM in BALB/c mice. A, Scheme of the subcutaneous immunization protocol and analysis of induced systemic responses. B and C, Cytokine production (B) or cytokine ratios (C) by splenocytes from mice immunized subcutaneously with diluent (Ctrl -), alum, PM or PM and alum, and stimulated in vitro with native grass pollen extract. D, Percentage of $CD4^+CD25^{high}FOXP3^+$ Treg cells in spleens of mice immunized with diluent (Ctrl -), alum, PM or PM and alum. Representative dot plots are shown. E and F, Serum grass pollen-specific antibodies (E) or ratio of serum grass pollen-specific IgG1/IgG2a (F) from mice immunized with diluent (Ctrl -), alum, PM or PM and alum. G, Inhibition of serum IgE binding to native grass pollen allergens by blocking antibodies from mice immunized with diluent (Ctrl -), alum, PM or PM and alum (mean ± SEM of n = 5 grass pollen allergic patients). For panels B-F, values are mean ± SEM of 18 individual mice per condition of 3 independent experiments. Unpaired Student *t* test: **P* < .05, ***P* < .01 and ****P* < .001

suggesting that alum did not alter glucose uptake but its final metabolic fate. To test whether PM alone or in combination with alum affect mitochondrial activity in hmoDCs, we monitored changes in mitochondrial membrane potential ($\Delta\psi$) with MitoTracker Red CMXRos. The mitochondrial $\Delta\psi$ was significantly increased by PM plus alum (Figure 5E), suggesting that alum has influence on the glucose metabolic fate by favouring mitochondrial oxidative phosphorylation. Further analysis of the redox status showed that the NADH accumulation induced by PM in hmoDCs was significantly reduced in the presence of alum (Figure 5F). Consequently, the $NAD^+$/NADH ratio was significantly higher in hmoDCs stimulated with PM plus alum than with PM alone (Figure 5G). Next, we studied the potential implication of mTOR pathway in the observed effects as a key regulator of metabolic responses. PM induced the rapid activation of mTOR pathway in hmoDCs as determined by the phosphorylation of its downstream substrate p70S6 kinase (Thr389) and its upstream activator Akt (Ser473) (Figure 5H,I). The phosphorylation of both p70S6 kinase and Akt was inhibited when hmoDCs were activated by PM plus alum (Figure 5H,I), indicating that alum inhibits the PM-induced activation of mTOR. Supporting these data, alum also inhibited the phosphorylation of phosphate and

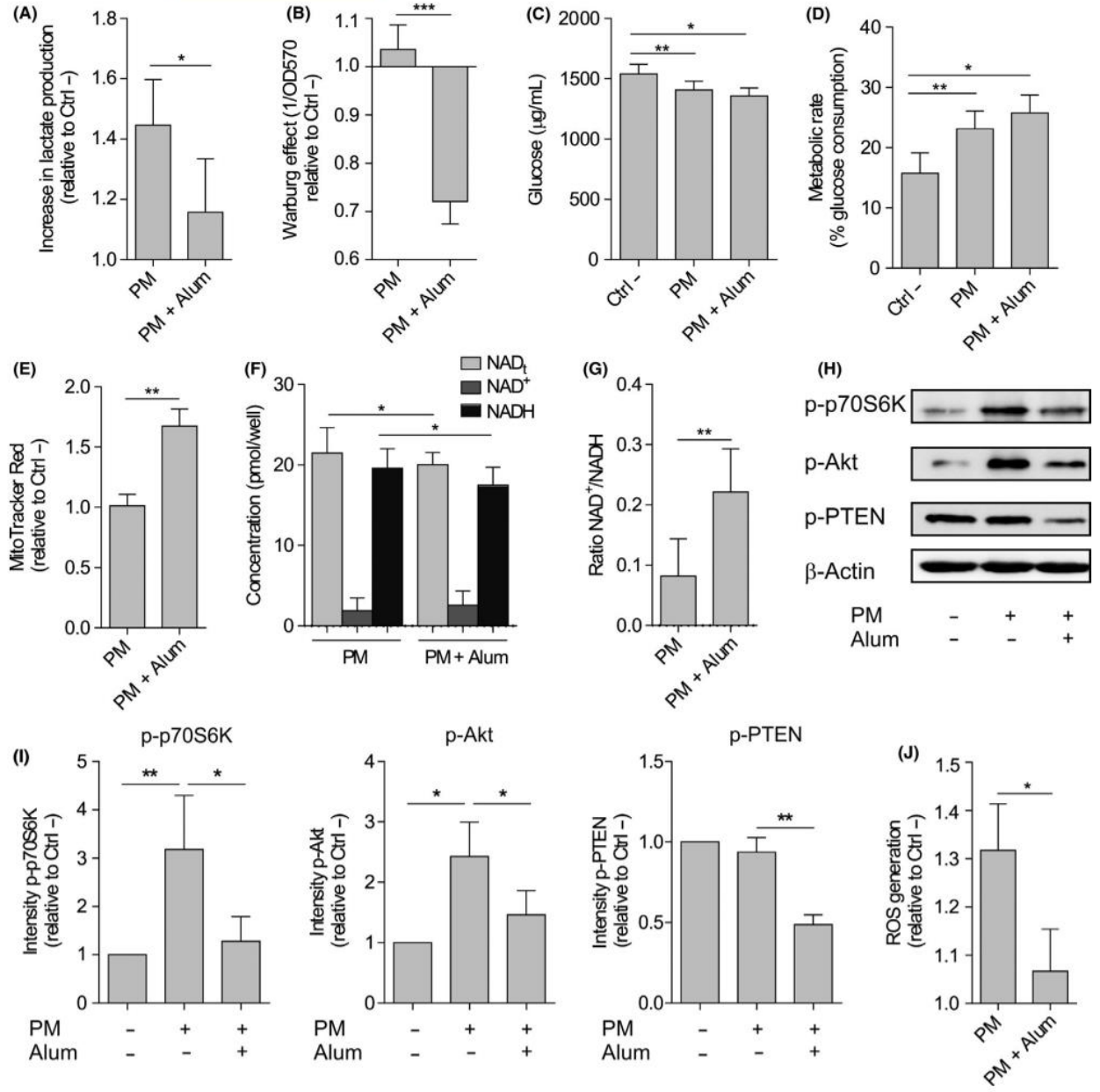



**FIGURE 5** Alum alters the metabolic reprogramming induced by PM in human DCs from healthy donors. A, Lactate content in cell-free supernatants from PM- or PM and alum-stimulated hmoDCs after 18 h relative to the unstimulated (Ctrl -) condition (n = 6). B, Quantification of the induced Warburg effect relative to the unstimulated (Ctrl -) condition (n = 6). C, Glucose consumption by stimulated DCs and (D) calculated metabolic rate (n = 6). E, Fluorescence intensity of stimulated hmoDCs stained with MitoTracker Red (n = 5). F, Cellular redox status analysed based on the determination of $NAD^+$, NADH and total NAD levels (NADt) (n = 5). G, $NAD^+$/NADH ratios. H, Western blot analysis of protein extracts from hmoDCs stimulated for 30 min in the indicated conditions, and one representative example out of 4 is shown. I, Quantification of the reactive phosphorylated bands by scanning densitometry (mean ± SEM of n = 4 independent experiments). J, Intracellular ROS quantification in hmoDCs after 18 h of stimulation with PM or PM and alum (n = 6). Paired Student *t* test: $^*P < .05$, $^{**}P < .01$ and $^{***}P < .001$

tensin homologue (PTEN) (Ser380/Thr382/383), an inhibitor of mTOR signalling pathway that remains inactive while it is phosphorylated (Figure 5H,I). In addition to its role in glycolytic metabolism, previous studies showed a positive interplay between PI3K/Akt/mTOR signalling pathway and the regulation of ROS production.[25] PM treatment significantly increased ROS production in hmoDCs, which was significantly inhibited in the presence of alum (Figure 5J). Collectively, our results demonstrate that alum impairs the PM-induced activation of mTOR and metabolic reprogramming in hmoDCs.

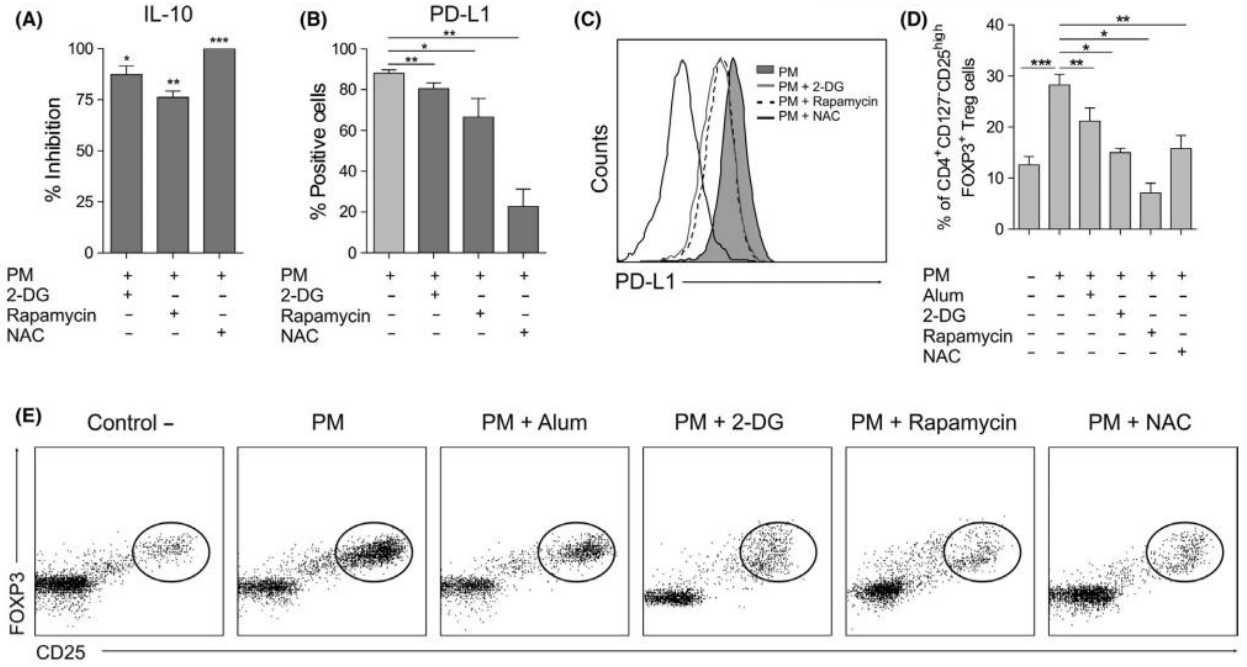



**FIGURE 6** Influence of mTOR pathway, glycolysis and ROS production in the induction of human tolerogenic DCs by PM. A-C, IL-10 production (n = 6; A), percentage of hmoDCs expressing PD-L1 (n = 6; B) and flow cytometry representative histogram of PD-L1 expression (C) of hmoDCs after stimulation with PM for 18 h in the presence of 2-DG, NAC or rapamycin. D, $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells generated after 5 d by allogeneic hmoDCs stimulated in the indicated conditions (gating in lymphocytes, n = 5). E, Flow cytometry representative dot plots of the generated $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells under the indicated conditions. Paired Student *t* test: $^*P < .05$, $^{**}P < .01$ and $^{***}P < .001$

### 3.6 | Glycolytic metabolism, mTOR activation and ROS production contribute to the tolerogenic properties imprinted by PM in hmoDCs that are impaired by alum

The inhibition of glycolysis with 2-DG, mTOR with rapamycin and ROS activity with NAC in PM-activated hmoDCs significantly suppressed the production of the anti-inflammatory cytokine IL-10 (Figure 6A). Similarly, the inhibition of glycolysis, mTOR and ROS activity significantly decreased the expression of PD-L1 in PM-activated hmoDCs (Figure 6B). Representative histograms of PD-L1 expression in PM-activated hmoDCs under the different conditions are displayed (Figure 6C). To assess the relevance of these findings in the generation of $FOXP3^+$ Treg cells by PM, we performed coculture experiments with PM-activated hmoDCs in the presence of the different inhibitors. As expected by the previous experiments, alum significantly reduced the frequency of PM-induced $FOXP3^+$ Treg cells (Figure 6D). Similarly, inhibition of glycolysis with 2-DG, mTOR with rapamycin and ROS activity with NAC in PM-activated hmoDCs significantly reduced the percentage of induced $FOXP3^+$ Treg cells (Figure 6D,E). These data suggest that (i) these pathways contribute to the induction of $FOXP3^+$ Treg cells by PM-activated DCs and (ii) alum impairs such induction through its inhibitory effect on these signalling pathways.

## 4 | DISCUSSION

In this study, we show that alum impairs the tolerogenic properties imprinted by allergoids conjugated to nonoxidized mannan. Alum decreases the expression of PD-L1 and IL-10 production in PM-activated human DCs, reducing their capacity to generate functional $FOXP3^+$ Treg cells. Mechanistically, we demonstrated that alum inhibits mTOR activation, shifts glycolysis metabolism and reduces ROS production in human PM-activated DCs, thus impairing the tolerogenic properties promoted by PM. Subcutaneous immunizations of mice with PM adsorbed to alum induces a shift to allergic responses and impairs the induction of splenic $FOXP3^+$ Treg cells supporting the in vitro results with human cells. Remarkably, alum did not alter the capacity of PM to produce IgG-blocking antibodies with similar functional activity than those generated by PM in the absence of alum. Overall, our data point against the use of alum in order to preserve the tolerogenic properties imprinted by allergoids conjugated to mannan in DCs. This should be also taken into account when considering novel vaccination approaches aimed to induce tolerogenic DCs,[26,27] especially when the mTOR pathway is involved in such effects.[28] This might be also relevance for those vaccines based on triggering trained immunity, in which the Akt/mTOR activation is essential.[29,30]

This is a paradigmatic study revealing the importance of the better understanding of the molecular mechanisms driving alum

adjuvanticity in the context of allergy, especially in humans, to continue improving allergen vaccine formulations for AIT. Allergoid-mannan conjugates represent a major development in the search of novel vaccine approaches to improve AIT,[6,9-11] and Alum was introduced as a vaccine adjuvant in 1926 by Glenny et al,[31] and since 1937, it is used in AIT. Although there are different adjuvant preparations that can be used in AIT as immunostimulants (ie, mineral salts or TLR-targeting adjuvants) or delivery systems (ie, different types of micro- and nanoparticles),[11] nowadays most of the subcutaneous vaccines for AIT include alum as adjuvant.[15] Although it is considered safe in terms of acute local or systemic effects,[18] the molecular mechanisms driving alum adjuvanticity are not completely understood.[20] Alum polarizes responses into Th2 cells in mice, whereas in humans there is still controversy.[32-34] Recent findings showed that alum can also activate innate immune responses in DCs and macrophages.[19,20] Here, we performed a comprehensive immunologic study of the potential influence of alum in the tolerogenic effects imprinted by PM targeting CLRs in DCs.[9] Alum significantly decreases the production of IL-10 and PD-L1 expression in human PM-activated DCs, which are molecules involved in the generation of functional Treg cells.[35,36] In contrast, the OX40-L expression, a molecule favouring Th2 polarization,[37,38] is significantly increased in the presence of alum. At the T-cell level, we showed that in humans, alum (in the presence of PM) not only promotes Th2 responses but also a broad pro-inflammatory profile, with Th1 and Th17 cells being also enhanced, whereas inhibiting the generation of functional FOXP3$^+$ Treg cells. Up to now, no data showing cross-talk between CLR- and alum-mediated signalling pathways have been reported. Alum is sensed by membrane lipids on DCs inducing activation in a receptor-independent manner that is mediated by the Syk-PI3K pathway.[20,39] PM trigger CLRs such as Syk-coupled Dectin-2 or DC-SIGN, both involved in PD-L1 expression and cytokine signature in human PM-activated DCs.[9] Our data clearly suggest that alum interferes with the CLR-mediated signalling pathways activated by PM in DCs.

Metabolic reprogramming plays a very important role in the control of DCs' function by regulating tolerogenicity *vs.* immunogenicity.[21,22] mTOR is a central regulator of cell metabolism, growth, proliferation and survival.[40,41] To gain insight into potential novel molecular mechanisms involved in the observed effects, we studied metabolic changes induced by PM in DCs and how alum could interfere on them. PM rapidly activates mTOR signalling pathway and PM-activated DCs display a high rate of glycolysis and lactic acid fermentation (Warburg effect),[40,42] features that were impaired by alum. Increased mTOR pathway activity is correlated with enhanced glycolysis, which appears to be associated with the generation of peripherally induced Treg cells.[21] Lactate has been also shown to enhance IL-10 production in macrophages and DCs.[43] It is well-recognized that TLR activation in DCs and macrophages induces a glycolytic burst,[44,45] but data for CLRs are mostly limited to Dectin-1 activated by β-glucan.[29] We demonstrate that mTOR activation is implicated in the induction of IL-10 and PD-L1 in human PM-activated DCs. Schülke *et al* also described that in mDCs activated by the fusion protein rFlaA:Betv1, mTOR regulates IL-10 production.[28] Similarly, the expression of PD-L1, a key molecule for the induction of functional FOXP3$^+$ Treg cells,[36,46,47] has been shown to be regulated by mTOR in DCs.[48] Herein, we show that the inhibition of PM-induced mTOR signalling and the reduction in PD-L1 expression in DCs by alum correlate with a reduction on the generation of FOXP3$^+$ Treg cells. Supporting this view, SH2 domain-containing inositol 5'-phosphatase (SHIP)-deficient mice, in which the Akt/mTOR pathway is upregulated on myeloid cells,[49] show a remarkable increase in FOXP3$^+$ Treg cells.[50]

Tolerogenic DCs display enhanced glycolytic capacity and ROS production with respect to mature pro-inflammatory DCs.[51] PM significantly increases intracellular ROS in human DCs, which were inhibited by alum. Recently, D-mannose treatment increased ROS production in T cells compared with TCR stimulation alone.[52] In line with our results, blockade of ROS activity by NAC significantly reduced numbers of D-mannose-induced Treg cells.[52] Although ROS have been considered toxic products of cellular metabolism, increasing evidence supports the idea that low amounts of ROS are positive contributors to normal signalling pathways.[42,53] Previous studies demonstrated that ROS further activates Akt/mTOR pathway, enabling optimal T-cell proliferation and glycolysis.[53-55] Therefore, our results confirm that the inhibition of ROS production and the blockade of mTOR signalling by alum in PM-activated DCs represent novel mechanisms by which this adjuvant impairs the induction of Treg cells.

Interestingly, mice immunized with PM in the presence of alum display significantly lower numbers of splenic FOXP3$^+$ Treg cells and higher IFN-γ, IL-5 and IL-4 levels than mice immunized only with PM. The ratio of serum IgG1/IgG2a is significantly higher in mice immunized with PM plus alum, indicating that alum also impairs in vivo tolerogenic responses to PM. Remarkably, alum did not enhance the capacity of PM to induce allergen-specific antibodies with blocking activity, indicating that the absence of alum in PM vaccine formulations would not significantly modify this feature.

In conclusion, this study provides novel insights into molecular pathways that might be affected by alum as adjuvant in AIT. We uncover novel molecular mechanisms involving mTOR, glycolysis and ROS production by which alum interferes with CLR-mediated signalling pathways activated by polymerized allergoids conjugated to nonoxidized mannan in human DCs, thus impairing the imprinted tolerogenic properties. This study demonstrates the importance of understanding the influence of adjuvants such as alum in novel vaccine formulations for AIT. Future studies on how other approved adjuvants for AIT might influence the tolerogenic features imprinted by allergoids conjugated to mannan could also contribute to provide novel mechanistic insights into their way of action. Finally, our findings might well go beyond AIT formulations and could be also relevant for other types of vaccines such as those promoting innate trained immunity, in which mTOR activation and metabolic reprogramming represent key mechanistic events in their mode of action.

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## CONFLICTS OF INTEREST

OP has received payment for lectures and participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, Inmunotek SL, Novartis, Sanofi Genzyme and Stallergenes. OP has received research grants from Inmunotek SL and Novartis SL. JLS is the founder and CEO of Inmunotek SL. EF-C. is a shareholder and employee of Inmunotek SL. IS is an employee of Inmunotek. The rest of the authors declare no competing financial interests.

## AUTHOR CONTRIBUTIONS

OP and JLS conceived and designed the study. C. B.-V. (human experiments), IS (mice experiments) and M. P.-D. (technical support for human experiments) performed the experiments. E. F.-C, JLS and O.P provided reagents. C. B.-V., IS, M. P.-D., EF-C., JLS and O.P analysed and discussed the data. OP and C. B.-V wrote the paper. All the authors read and approved the final version of the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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# ONLINE REPOSITORY

## METHODS

### HmoDCs generation, naive CD4⁺ T cells purification and total blood DCs isolation

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors or allergic patients by Ficoll density gradient centrifugation (800g, 20 minutes). Immature hmoDCs were generated from blood monocytes obtained from total PBMCs using anti-CD14 microbeads and cultured for 6 days with RPMI medium containing 100 ng/mL of IL-4 and GM-CSF (PeproTeck, Rocky Hill, NJ). The purity and phenotype of monocytes and generated immature hmoDCs were analysed 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) and total dendritic cell fraction was obtained from PBMC with ‘‘Blood Dendritic Cell Isolation Kit II’’ (Miltenyi Biotec), according to manufacturer’s protocol.

PBMCs from healthy donors or allergic patients were stimulated with medium (Ctrl -), PM (50 µg/mL) or PM with alum (0.1 mg/mL) for 3 days. Cell-free supernatants were used to quantify IL-10, IL-5, IFN-γ and IL-17A by ELISA.

### Flow cytometry

Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 minutes at room temperature in the darkness. The corresponding isotype controls were included in each staining (IgG2A-FITC, IgG1-PE, IgG2A-PerCP, or IgG1-APC). Flow cytometric monoclonal antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany): human anti-HLA-DR-fluorescein isothiocyanate [FITC], anti–HLA-DR–allophycocyanin [APC], anti-CD83–APC, anti-CD303–phycoerythrin [PE], anti-CD1c–FITC, anti-CD127–PE, FcR Blocking; BioLegend (San Diego, CA): human anti-FOXP3–Alexa Fluor 488, anti-CD25–APC, anti-CD4–peridinin chlorophyll protein complex [PerCP], anti-IL-10-PE, anti-IL-17A-PE, anti-IL-5-PE, mouse anti-FOXP3–Alexa Fluor 488, mouse anti-CD4–PerCP, and mouse anti-CD25–PE; and BD PharMingen (San Jose, CA): human anti-PD-L1–FITC, anti-OX40L–PE, anti-CD3–APC, anti-CD19–PE/Cy7, and anti-IFNγ–Alexa Fluor 488. For analysis of FOXP3 expression in human T cells primed with DCs, cells were first subjected to surface staining with anti-human CD4-PerCP, CD127-PE, and CD25-APC antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488, according to manufacturer’s recommendations. The same protocol described above was carried out for the phenotypic characterization of $CD4^+CD25^{high}FOXP3^+$ Treg cells in freshly isolated mouse

splenocytes using anti-mouse CD4-PerCP and CD25-PE antibodies for surface staining and anti-mouse FOXP3-Alexa 488 for intranuclear staining. The corresponding isotype controls were included in each staining. Flow cytometry analysis was performed in a FACSCalibur cytometer (Beckton Dickinson) in the Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

**Cytokine quantification**

Concentrations of IL-6, IL-12p70, IL-4, IL-10, IFN-γ, and IL-5 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, CA). IL-23 and IL-17A levels were quantified by Human IL-23 ELISA Ready-SET-Go! (e-Biosciences, San Diego, CA) and quantikine Elisa Kit (RD Systems), respectively. In all cases, manufacturer's instructions were followed with minor modifications.

**Coculture experiments**

HmoDCs or human total blood DC enriched fraction treated with medium (Ctrl -), alum (0.1 mg/mL), PM (50 µg/mL) or the combination of PM and alum were cocultured with purified allogeneic naïve $CD4^+$ T cells (DC/T cell ratio of 1:5) for 5 days. IL-17A, IFN-γ, IL-5 and IL-10 were quantified in cell-free supernatants by ELISA. For intracellular cytokine production, the primed $CD4^+$ T cells were washed and restimulated with 25 ng/mL PMA plus 1 mg/mL ionomycin for 6 hours. 10 µg/mL brefeldin A was added during the last 4 hours. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Cells were stained with fluorochrome-labeled mAbs to IL-17A, IFN-γ, IL-5, and IL-10.

**Treg cell suppression assay**

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

**Metabolic studies**

The Warburg effect in stimulated hmoDC cultures was determined photometrically 18 hours after stimulation by quantifying the OD at 570 nm and calculating the Warburg effect as 1/OD570 normalized to the unstimulated. Glucose concentrations in 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 DCs (glucose concentration in RPMI 1640 = 2 mg/mL). Lactate concentrations in culture supernatants were determined 18 hours after stimulation by using the colorimetric L-Lactate Assay kit (Abcam), according to the manufacturer's recommendations. To measure mitochondrial membrane potential, MitoTracker Red CMXRos was used (250 nM, Thermo Fisher Scientific). Total $NAD^+$/nicotinamide adenine dinucleotide (NADH) content, $NAD^+$, and NADH concentrations in lysates of DCs were determined by using NAD/NADH Assay Kit (Abcam), according to the manufacters' recommendations. Reactive oxygen species were measured by DCFDA Cellular ROS Detection Assay Kit (Abcam, Cambridge, United Kingdom) following manufacturer's instructions. Briefly, total ROS levels were measured using the cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA). Cells were stained with DCDFA for 30 minutes at 37°C after 18 hours of treatment with the corresponding stimuli. Fluorescence was measured in a FLUOstar Omega microplate reader (Ex/Em = 485/535 nm).

**Western blotting**

Freshly isolated hmoDCs were treated with medium (Ctrl -), PM (50 µg/mL) and PM previously adsorbed to alum (0.1 mg/mL). After 30 minutes at 37°C, cells were harvested and lysed with RIPA buffer (ThermoFisher scientific, Madrid, Spain) supplemented with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling, Danvers, Mass) for 30 minutes at 4°C with vortexing every 10 minutes. Lysates were centrifuged for 15 minutes at 10,000g and 4°C, and supernatants were collected for protein quantification with Micro BCA Kit (Pierce, Rockford, Ill). Ten micrograms of total protein from cell lysates was separated by means of SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif). The membrane was incubated with the indicated antibodies used as follows: phospho-PTEN (Ser380/Thr382/383) (1:1000, Cell Signaling), phospho-p70 S6 Kinase (Thr389) (1:500; Cell Signaling), phospho-Akt (Ser473) (1:1000; Cell Signaling) and β-actin (1:15000, Sigma) as primary antibodies and 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 multigauge software, and values were expressed relative to the β-actin loading control.

**Immunization of mice with control, alum, PM, or PM with alum: quantification of induced serum allergen-specific immunoglobulins levels; and cytokine profiles of splenocytes stimulated in vitro with N**

BALB/c female mice (6 weeks old) were immunized 3 times with 20 µg (200 µL at 100 µg/mL) of PM, or PM previously adsorbed to 2 mg of alum by means of subcutaneous injection every 15 days and killed 7 days after the last immunization. Serum specific IgG2a, IgG1, IgE reactivities to N induced with each treatment were determined by means of ELISA. Spleens were used to prepare single-cell suspensions according to conventional protocols. The presence of splenic $CD4^{+}CD25^{high}FOXP3^{+}$ Treg cells in the different immunized mice was quantified, as described above. Splenocytes were stimulated *in vitro* with N, and cytokine (IFN-γ, IL-5, IL-4 and IL-10) production was measured by using CBA Cytometric kits (Beckton Dickinson) in 48-hour culture supernatants. Animal experiments were approved by the Ethics Committee of Hospital Clínico San Carlos.

**ELISA inhibition of the binding of serum IgE from allergic patients to N extract by blocking antibodies induced after immunization of mice.**

The blocking capacity of the serum antibodies generated by means of immunization of mice with control, alum, PM, and PM with alum was analysed by using ELISA inhibition assays. Microtiter plates (Corning Inc, Corning, NY) were coated with 10 µg of N in 100 µl of PBS overnight at 4°C. Plates were washed 3 times with PBS and 0.1% vol/vol Tween-20 and blocked for 1 hour with PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. Then plates were incubated with the serum of mice immunized against negative control, alum, PM, or PM with alum extracts. After 1 hour of incubation, plates were washed 4 times with PBS and 0.1% vol/vol Tween-20 and incubated for 2 hours with individual serum from 5 patients with well-defined grass pollen allergy diluted 1:50 in PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. The patients were previously described[1] and the study was approved by the Regional Ethics Committee of Comunidad Autónoma de Madrid (EudraCT: 2014-005471-88). After 4 washes, bound IgE antibodies were detected by incubating for 1 hour with biotin anti-human IgE rabbit monoclonal antibody (1:100000 diluted) (RevMab Biosciences, South San Francisco, CA), followed by 45 minutes with horseradish peroxidase–coupled streptavidin (diluted 1:2500). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% $H_2O_2$ and 0.63 mg/mL *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0),

and the reaction was stopped with 3 N $H_2SO_4$. OD was measured at 492 nm in an ELISA reader.

## LEGEND TO SUPPLEMENTARY FIGURES

**FIGURE S1.** Suppression capacity of FOXP3$^+$ Treg cells induced by allogeneic PM or PM and alum-treated hmoDCs. (A) Representative dot plots of the purification of Treg cells by sorting. (B) and (C) Proliferation of CFSE-labeled PBMCs gated on CD4$^+$ cells after 5 days of coculture with autologous purified FOXP3$^+$ Treg cells induced by allogeneic PM-treated (Fig. S1B) or PM and alum-treated (Fig. S1C) hmoDCs. The percentages of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition at different Treg cell/responder PBMC ratios are shown (n = 2).

**FIGURE S2.** Effects of alum in hmoDCs and PBMCs from allergic patients. (A) Percentage of induced CD4$^+$CD25$^{high}$CD127$^-$FOXP3$^+$ Treg cells and cytokine ratios produced by T cells primed by allogeneic PM or PM and alum-treated hmoDCs from allergic patients after 5 days (n = 3). (B) Cytokines and cytokine ratios produced by PBMCs from allergic patients stimulated with the indicated stimulus for 3 days (n = 3). (C) Cytokines and cytokine ratios produced by PBMCs from non-allergic donors stimulated with the indicated stimulus for 3 days (n = 3).

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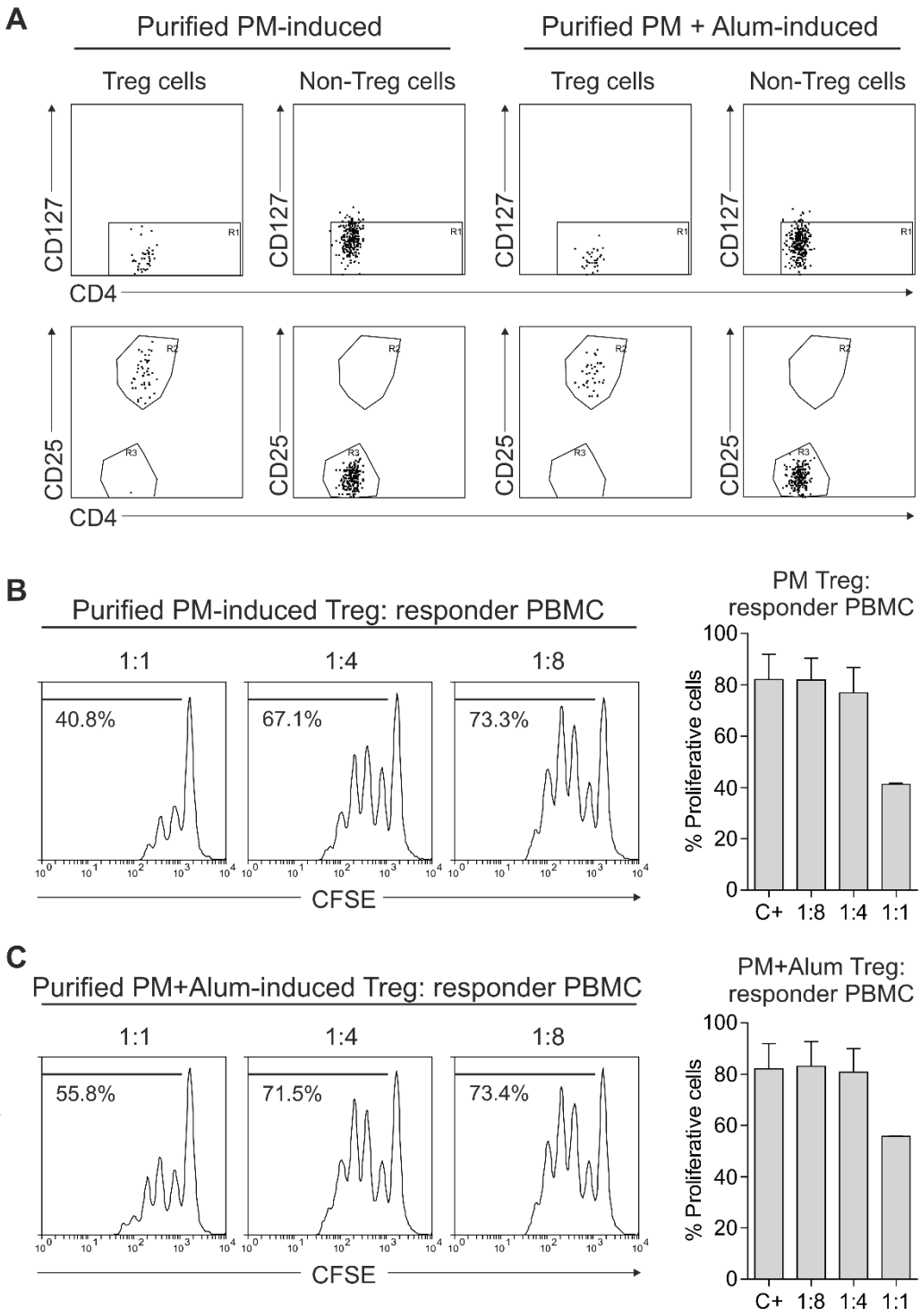


Figure supplementary 1. Benito-Villalvilla et al.

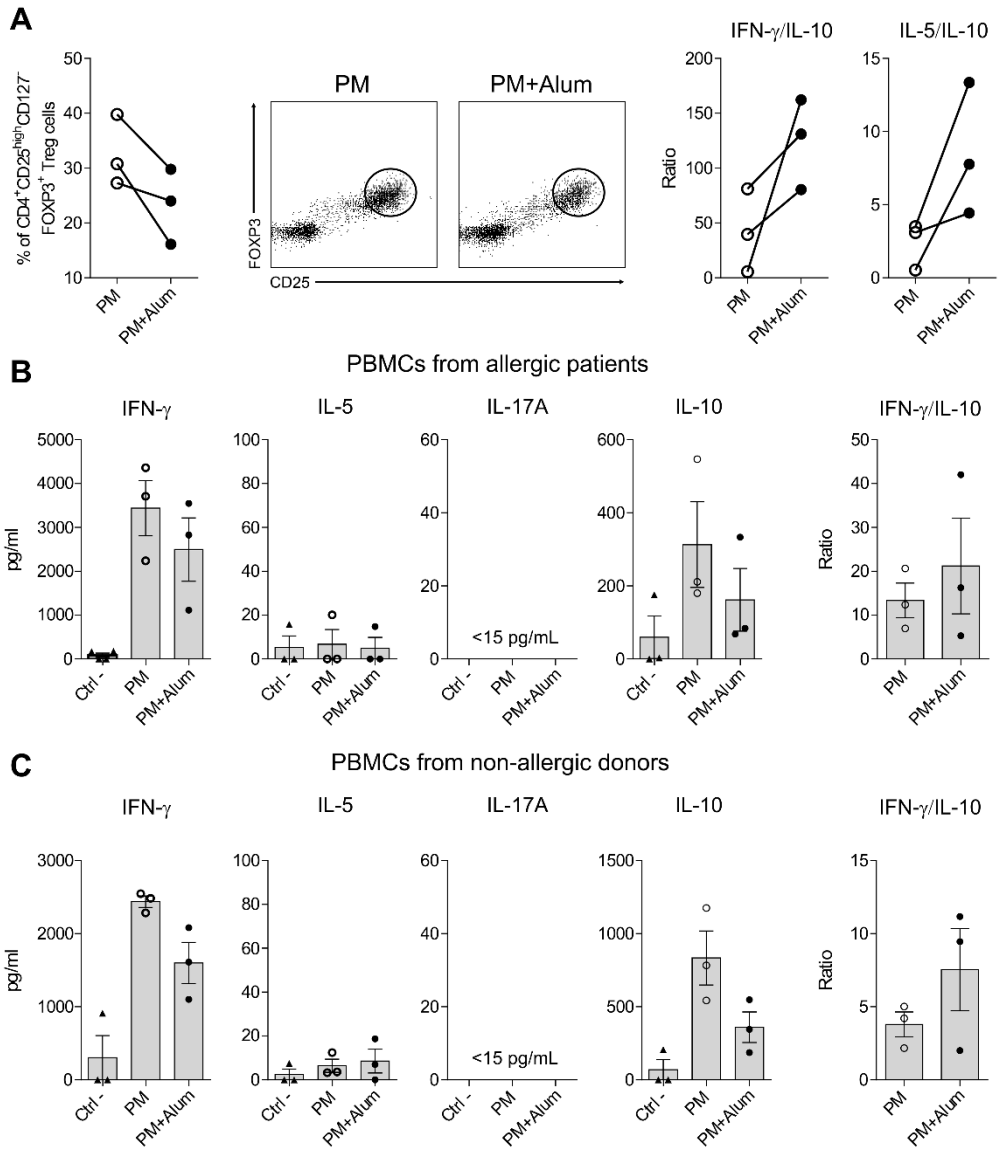


Figure supplementary 2. Benito-Villalvilla et al.

# ARTICLE II: Allergoid-mannan conjugates reprogram monocytes into tolerogenic dendritic cells via epigenetic and metabolic rewiring

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Monocytes are one of the first cell types migrating into the areas of vaccine administration, and can be differentiated into immunogenic or tolerogenic DCs. To study whether allergoid-mannan conjugates could reprogram monocyte differentiation into DCs, the phenotype and function of DCs from nonatopic or allergic subjects differentiated in the absence or presence of allergoid-mannan conjugates were compared. HmoDCs differentiated in the presence of allergoid-mannan conjugates (mannan-tolDCs) displayed a stable tolerogenic profile characterized by a lower cytokine response to LPS-stimulation, higher expression of tolerogenic markers as IDO or PD-L1, and a higher capacity to induce the generation of functional $FOXP3^+$ Treg cells. Regarding the molecular mechanisms underlying these effects, a shift in the glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS was observed in mannan-tolDCs compared to conventional DCs. Besides, mannan-tolDCs displayed epigenetic rewiring with modifications in specific histone marks, lower expression of histone deacetylase genes and change in the expression of miRNAs. These results increase the knowledge about the mechanisms of a novel AIT, which can contribute to the induction of allergen-tolerance by other AIT vaccines.

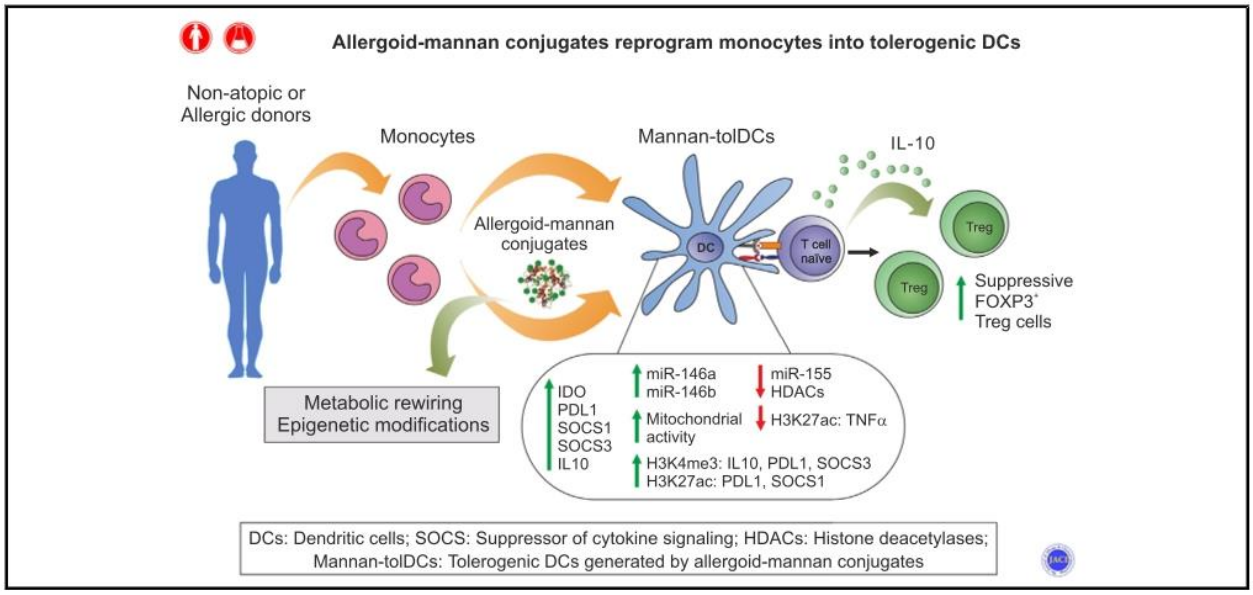
*Herein, this study is presented with the signed consent of all the co-authors. During the research, I conducted all mechanistic experiments and allergic patient's samples experiments, and participated in the experimental design, the analysis and discussion of the data, and also in the organization, writing and revision of the manuscript.*

ARTICLE IN PRESS

# Allergoid–mannan conjugates reprogram monocytes into tolerogenic dendritic cells via epigenetic and metabolic rewiring

**Cristina Benito-Villalvilla, BSc,[a] Mario Pérez-Diego, BSc,[a] Alba Angelina, PhD,[a] Kai Kisand, PhD,[b] Ana Rebane, PhD,[b] José Luis Subiza, MD, PhD,[c] and Oscar Palomares, PhD[a]** *Madrid, Spain, and Tartu, Estonia*

**GRAPHICAL ABSTRACT**




**Background: Allergoid–mannan conjugates are novel vaccines for allergen-specific immunotherapy being currently assayed in phase 2 clinical trials. Allergoid–mannan conjugates target dendritic cells (DCs) and generate functional forkhead box P3 (FOXP3)-positive Treg cells, but their capacity to reprogram monocyte differentiation remains unknown.**

**Objective: We studied whether allergoid–mannan conjugates could reprogram monocyte differentiation into tolerogenic DCs and the underlying molecular mechanisms.**

**Methods: Monocytes from nonatopic and allergic subjects were differentiated into DCs under conventional protocols in the absence or presence of allergoid–mannan conjugates. ELISA, real-time quantitative PCR, coculture, flow cytometry, and suppression assay were performed. Metabolic and epigenetic techniques were also used.**

**Results: Monocyte differentiation from nonatopic and allergic subjects into DCs in the presence of allergoid–mannan conjugates yields stable tolerogenic DCs. Lipopolysaccharide-stimulated mannan-tolDCs show a significantly lower cytokine production, lower TNF-α/IL-10 ratio, and higher expression of the tolerogenic molecules *PDL1, IDO, SOCS1, SOCS3*, and *IL10;* and they induce higher numbers of functional FOXP3$^+$ Treg cells than conventional DC counterparts. Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation. They also display epigenetic reprogramming involving specific histone marks**

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Disclosure of potential conflict of interest: O. Palomares has received fees for lectures and/or participation in Advisory Boards from Allergy Therapeutics, Amgen, AstraZeneca, Diater, GSK, Inmunotek SL, Novartis, Sanofi-Genezyme, Regeneron, and Stallergenes; and has received research grants from Inmunotek SL and Novartis SL. J. L. Subiza is the founder and CEO of Inmunotek SL. The rest of the authors declare that they have no relevant conflicts of interest.

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**within tolerogenic loci and lower expression levels of histone deacetylase genes. Mannan-tolDCs significantly increase the expression of the anti-inflammatory miRNA-146a/b and decrease proinflammatory miRNA-155.**
**Conclusions: Allergoid–mannan conjugates reprogram monocyte differentiation into stable tolerogenic DCs via epigenetic and metabolic reprogramming. Our findings shed light on the novel mechanisms by which allergoid–mannan conjugates might contribute to allergen tolerance induction during allergen-specific immunotherapy. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)**

***Key words:*** *Allergen-specific immunotherapy, allergoid–mannan conjugates, monocytes, tolerogenic dendritic cells, regulatory T cells, metabolism, epigenetics*

Allergen-specific immunotherapy (AIT) is currently the only disease-modifying treatment with potential curative capacity for allergy.[1,2] A better understanding of the immune mechanisms involved in allergen tolerance induction during AIT is of paramount importance, not only to design safer and more effective AIT vaccines but also to identify potential novel biomarkers for managing allergic patients undergoing treatment.[3,4] After rapid desensitization of effector cells, the immunologic changes associated with successful AIT include the generation and maintenance of functional allergen-specific Treg cells, which are promoted by the restoration of tolerogenic dendritic cells (DCs).[5,6] This is also linked to the induction of B regulatory cells secreting allergen-specific blocking IgG antibodies that neutralize allergens preventing degranulation of mast cells, basophils, and eosinophils and that also inhibit IgE-facilitated allergen presentation by antigen-presenting cells, thus impairing the activation of allergen-specific memory $T_H2$ responses.[3,7] Although data about the role of monocytes and other innate cells in the context of AIT are still limited, it has been recently shown that after the first year of AIT, increased numbers of intermediate monocytes in parallel with reduced numbers of nonclassical monocytes are observed.[8]

Although AIT is a successful treatment for many allergic patients, it still remains underused as a result of several drawbacks in terms of safety, efficacy, long therapy duration, and patient adherence. To overcome these issues, new vaccines using novel adjuvants and/or the use of alternative routes of administration are being developed.[9-11] Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan (allergoid–mannan conjugates) by an alternative single-step production approach represent next-generation AIT vaccines targeting DCs.[12,13] Allergoid–mannan conjugates are captured by DCs by mechanisms depending on mannose receptor and DC-SIGN (specific intercellular adhesion molecule-3-grabbing non-integrin)–mediated internalization. Allergoid–mannan conjugates induce blocking antibodies and promote the generation of human and mice functional forkhead box 3 (FOXP3)-positive Treg cells through programmed death ligand 1 (PD-L1) both *in vitro* and *in vivo*.[14,15] Its molecular mechanism of action on human DCs involve mTOR, glycolysis, and reactive oxygen species (ROS) production, and the presence of alum interferes with those signaling pathways, impairing the imprinted tolerogenic properties.[16] Subcutaneous administration of *Dermatophagoides farinae* allergoid–mannan conjugates in dogs with atopic dermatitis demonstrated a rapid clinical improvement within the first 3 months of treatment.[17] In humans, four phase 2 clinical trials, for grass pollen, birch pollen, and mite allergy (EudraCT nos. 2014-005471-88, 2015-000820-27, 2018-002522-23, and 2020-004126-32), have been performed or are currently ongoing.

Despite these mechanistic and clinical advances, whether allergoid–mannan conjugates are able to reprogram human monocyte differentiation into tolerogenic DCs and the underlying molecular mechanisms remain unknown. Monocytes are one of the first immune cells to be recruited to vaccine administration sites, where they might differentiate into immunogenic or tolerogenic DCs, depending on the signal they encounter.[18-22] Different studies have shown that mechanisms involving epigenetic and metabolic reprogramming drive the differentiation of human monocyte–derived DCs (hmoDCs) into immunogenic or tolerogenic DCs.[20,23,24]

We investigated whether allergoid–mannan conjugates could influence the differentiation of human monocytes into tolerogenic DCs. The immunogenicity versus tolerogenicity in human DCs is finely regulated by metabolic and epigenetic reprogramming. Therefore, we also wanted to study whether these mechanisms might be underlying the phenotypic and functional features imprinted by allergoid–mannan conjugates during monocyte differentiation. We uncovered novel mechanisms by which allergoid–mannan conjugates could restore tolerance to allergens during AIT.

*Abbreviations used*

| | |
|---|---|
| AIT: | Allergen-specific immunotherapy |
| ChIP: | Chromatin immunoprecipitation |
| DC: | Dendritic cell |
| DC-SIGN: | Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin |
| FOXP3: | Forkhead box 3 |
| H3K27ac: | Histone H3 acetylation on lysine 27 |
| H3K27me3: | Histone H3 trimethylation on lysine 27 |
| H3K4me3: | Histone H3 trimethylation on lysine 4 |
| HDAC: | Histone deacetylase |
| hmoDC: | Human monocyte–derived dendritic cell |
| IDO: | Indoleamine 2,3-dioxygenase |
| LNA: | Locked nucleic acid |
| LPS: | Lipopolysaccharide |
| Mannan-tolDC: | Tolerogenic dendritic cell generated by allergoid–mannan conjugate |
| miRNA: | MicroRNA |
| mRNA: | Messenger RNA |
| MTA: | 5′-Methylthioadenosine |
| NADH: | Nicotinamide adenine dinucleotide |
| OXPHOS: | Oxidative phosphorylation |
| PD-L1: | Programmed death ligand 1 |
| ROS: | Reactive oxygen species |
| SOCS: | Suppressor of cytokine signaling |

## METHODS

### Material, media, and reagents

Glutaraldehyde-polymerized grass pollen (*Phleum pratense*) allergoids conjugated to nonoxidized mannan (allergoid–mannan conjugates) and mannan-free glutaraldehyde-polymerized grass pollen allergoids (allergoids) were provided by Inmunotek (Madrid, Spain). PBMCs for monocyte purification were obtained by Ficoll density gradient centrifugation ($800 \times g$, 20 minutes) from buffy coats obtained from Centro de Transfusiones

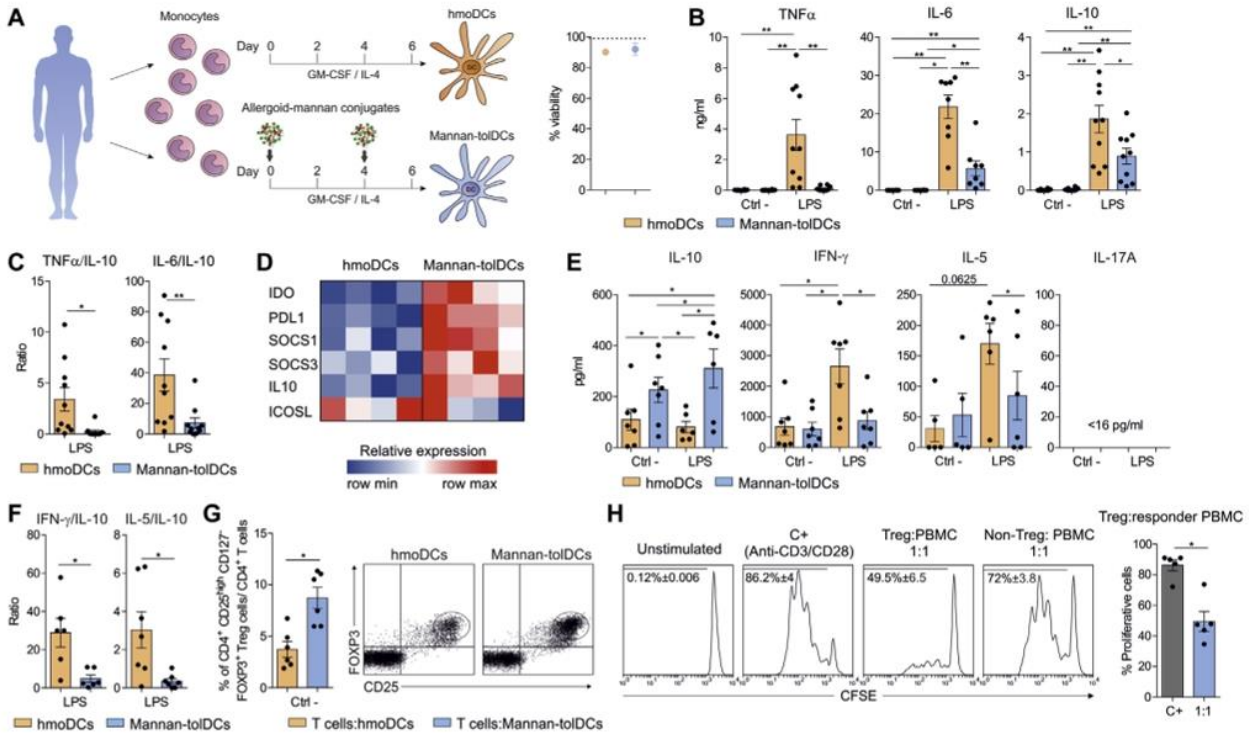


**FIG 1.** Mannan-tolDCs display tolerogenic features. **A,** Mannan-tolDCs generation protocol and viability of the generated cells. **B** and **C,** Cytokine (*B*) or cytokine ratios (*C*) produced in the presence of medium (Ctrl −) or LPS of conventional hmoDCs or mannan-tolDCs from nonatopic donors. **D,** Heat map analysis of mRNA expression of tolerogenic molecules by freshly isolated hmoDCs or mannan-tolDCs. **E** and **F,** Cytokines (*E*) or cytokine ratios (*F*) produced by allogeneic naive $CD4^+$ T cells primed by unstimulated or LPS-stimulated hmoDCs or mannan-tolDCs from nonatopic donors after 5 days. **G,** Percentage and representative dot plots of induced $CD4^+CD25^{high}CD127-FOXP3^+$ Treg cells by allogeneic unstimulated hmoDCs or mannan-tolDCs after 5 days (gating in lymphocytes). **H,** Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMCs gated on $CD4^+$ T cells after 5 days of coculture with autologous purified Treg cells and non-Treg cells generated by allogeneic unstimulated mannan-tolDCs (ratio 1:1). The percentage of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition is shown. Results are shown as means ± SEMs of 8-10 (*B* and *C*), 6-7 (*E* and *F*), 6 (*G*), and 5 (*H*) independent experiments. Wilcoxon test, $*P < .05$, $**P < .01$, and $***P < .001$.

de la Comunidad Autónoma de Madrid of healthy nonatopic donors (negative specific IgE against grass pollen and other common aeroallergens and total serum IgE levels <30 ng/mL as determined by ELISA). Grass pollen–allergic patients, whose demographic and clinical features are collected in Table E2 in this article's Online Repository at www.jacionline.org, had recurrent clinical symptoms during the grass pollen season and displayed specific IgE against grass pollen allergens. The 6 patients included in this study were previously clinically characterized.[14] The study was approved by the corresponding regional ethics committee of Centro de Transfusiones de la Comunidad Autónoma de Madrid (EudraCT no. 2014-005471-88). For the in vitro experiments we performed, fresh blood samples were drawn after receipt of informed consent.

## Statistical analysis

In all experiments, data represent the means ± SEMs of the indicated parameters. Statistical differences were determined with the paired or unpaired Student *t* test when data followed normal distribution or Wilcoxon matched-pairs test when they did not using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, Calif). Significance is indicated in each figure.

All procedures used in this study are fully described in this article's Methods section in the Online Repository at www.jacionline.org.

## RESULTS

### Monocyte-derived DCs differentiated in the presence of allergoid–mannan conjugates display tolerogenic features

To analyze whether allergoids conjugated to mannan could reprogram the differentiation of human monocyte–derived DCs (hmoDCs), purified blood monocytes from nonatopic donors were differentiated into DCs under conventional protocols in the absence or presence of allergoid–mannan conjugates without affecting cell viability (Fig 1, *A*, and see Fig E1 in this article's Online Repository at www.jacionline.org). Neither conventional hmoDCs nor DCs generated in the presence of allergoid–mannan conjugates (mannan-tolDCs) produced cytokines in the presence of medium (unstimulated condition, hereafter referred to Ctrl −) (Fig 1, *B*). After 18 hours of lipopolysaccharide (LPS) stimulation, mannan-tolDCs produced significantly lower levels of the proinflammatory cytokines TNF-α and IL-6 than hmoDCs (Fig 1, *B*). The production of IL-6 was still significant compared to control cultures, as also occurred, to a greater extent, for the anti-inflammatory IL-10 (Fig 1, *B*). Therefore,

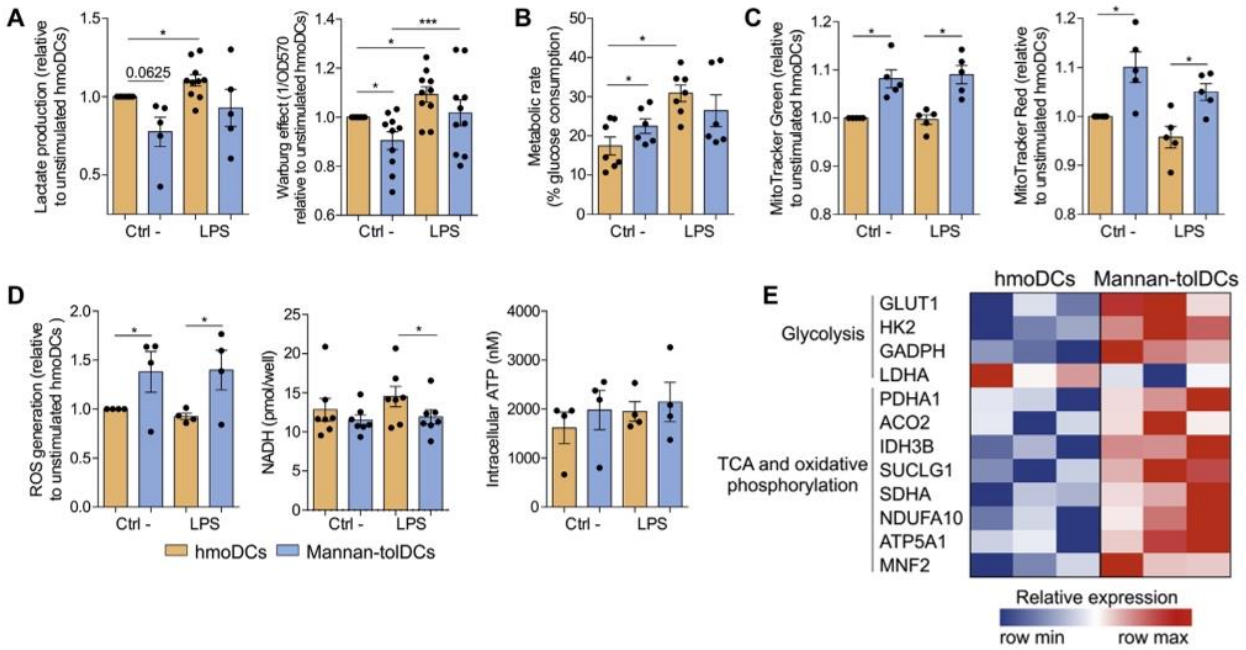


**FIG 2.** Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS. **A,** Quantification of the lactate content in cell-free supernatants with a lactate assay kit and the induced Warburg effect measured as 1/OD 570 nm of wells from unstimulated (Ctrl −) or LPS-stimulated hmoDCs and mannan-tolDCs after 18 hours relative to unstimulated hmoDCs. **B,** Metabolic rate induced after stimulation during 18 hours with media or LPS of conventional hmoDCs or mannan-tolDCs. **C,** Fluorescence intensity of cells stained with MitoTracker Green (mitochondrial mass) or MitoTracker Red (mitochondrial membrane potential) relative to unstimulated hmoDCs. **D,** Intracellular ROS, NADH, and ATP quantification. For NADH measures, well volume is 160 μL. **E,** Heat map of mRNA expression levels of genes in hmoDCs and mannan-tolDCs treated with LPS for 4 hours. Results are shown as means ± SEMs of 5-10 (*A*), 7 (*B*), 6 (*C*), and 4-8 (*D*) independent experiments. Paired Student *t* test or Wilcoxon test, $*P < .05$, $**P < .01$, and $***P < .001$.

the TNF-α/IL-10 and IL-6/IL-10 ratios were significantly lower in mannan-tolDCs than hmoDCs, suggesting that mannan-tolDCs display anti-inflammatory features (Fig 1, *C*). Freshly differentiated mannan-tolDCs expressed significantly higher messenger RNA (mRNA) levels of the classical tolerogenic molecules *IDO, PDL1, SOCS1, SOCS3,* and *IL10* than hmoDCs without differences for *ICOSL* (Fig 1, *D*). Remarkably, hmoDCs generated in the presence of allergoids without mannan conjugation (allergoids) displayed a cytokine signature similar to that observed for conventional hmoDCs upon LPS stimulation. In contrast, LPS-stimulated hmoDCs generated in the presence of mannan alone induce a similar cytokine profile than mannan-tolDCs (see Fig E2, *A,* in this article's Online Repository at www.jacionline.org). Allergoid DCs did not increase the mRNA expression levels of *IDO, PDL1, SOCS1, SOCS3,* or *IL10.* Mannan alone also induced the mRNA expression levels of the assayed tolerogenic markers (Fig E2, *B*), confirming that mannan is indispensable to imprint the anti-inflammatory and tolerogenic features observed in mannan-tolDCs. Supporting these data, LPS-stimulated mannan-tolDCs but not allergoid DCs showed decreased expression of CD86 and increased expression of PD-L1 compared to conventional hmoDCs (data not shown).

To assess the capacity of mannan-tolDCs and hmoDCs to polarize $CD4^+$ T-cell responses, we performed allogeneic cocultures. Unstimulated mannan-tolDCs generated T cells producing higher levels of IL-10 than T cells primed by unstimulated conventional DCs, without differences in IFN-γ and IL-5 production (Fig 1, *E*). Interestingly, LPS-stimulated mannan-tolDCs generated T cells producing significantly higher levels of IL-10 and still lower levels of IFN-γ and IL-5 than T cells generated by LPS-stimulated hmoDCs (Fig 1, *E*). IL-17A production was not detected in any assayed condition (Fig 1, *E*). The IFN-γ/IL-10 and IL-5/IL-10 ratios were significantly lower when T cells were primed by LPS-stimulated mannan-tolDCs than hmoDCs (Fig 1, *F*). Remarkably, mannan-tolDCs also induced significantly higher numbers of $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells than conventional hmoDCs (Fig 1, *G*). For functional experiments, we sorted the generated Treg cells ($CD4^+CD25^{high}CD127^-$) and non-Treg cells (see Fig E3, *A,* in this article's Online Repository at www.jacionline.org). Purified Treg cells generated by mannan-tolDCs, but not $CD4^+CD25^-CD127^-$ non-Treg cells, inhibited the proliferation of autologous PBMCs in a dose-dependent manner (Fig 1, *H,* and Fig E3, *B*), demonstrating that the induced Treg cells display functional suppressive capacity. Collectively, these data demonstrate that allergoids conjugated to mannan are able to reprogram monocyte differentiation and generate tolerogenic DCs able to prime functional Treg cells.

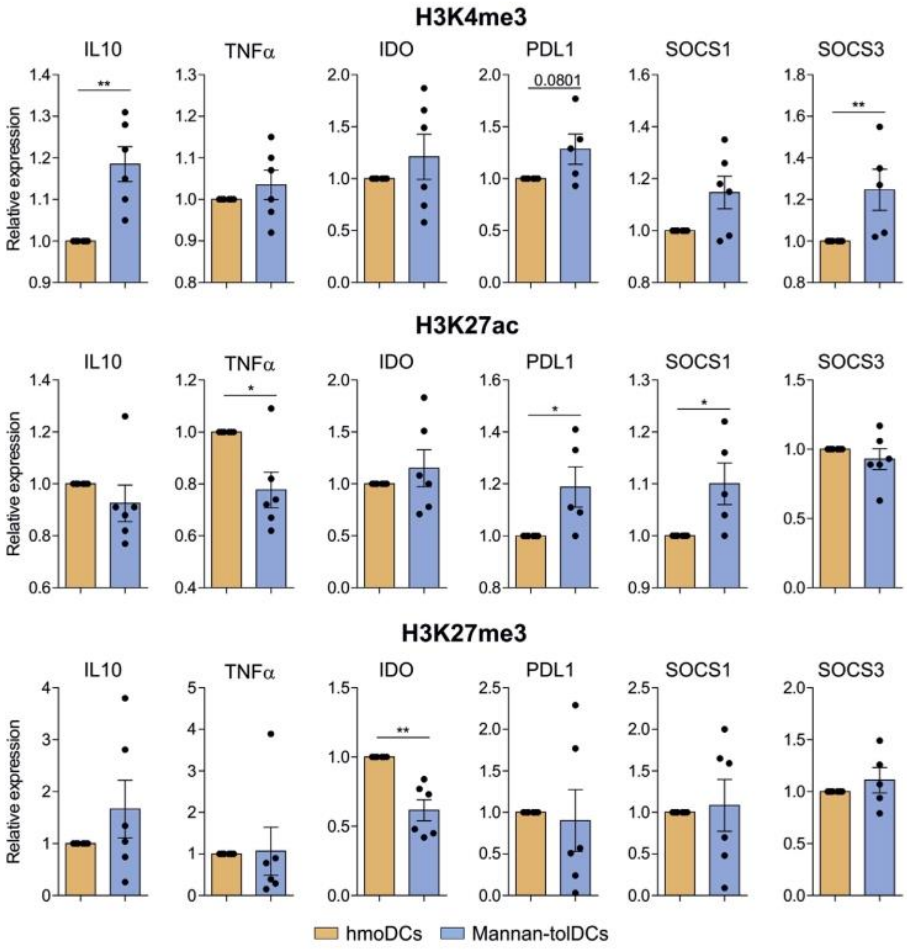


**FIG 3.** Mannan-tolDCs display epigenetic reprogramming involving specific histone marks associated with the activation of a tolerogenic transcriptional program. ChIP data of freshly hmoDCs and mannan-tolDCs was used to analyze the presence of H3K4me3, H3K27ac, and H3K27me3 histone modifications associated with the indicated molecules and cytokines. Results are shown as means ± SEMs of 6 independent experiments normalized to positive control gene *B2M* (for H3K4me3 and H3K27ac) or to negative control gene *MYOD1* (for H3K27me3). Mann-Whitney test, $*P < .05$, $**P < .01$, and $***P < .001$.

## Mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial oxidative phosphorylation

Immunogenicity versus tolerogenicity in human DCs is finely regulated by metabolic reprogramming.[25,26] Therefore, we studied changes in the metabolic state of the generated mannan-tolDCs compared to conventional hmoDCs. Unstimulated mannan-tolDCs displayed a significantly lower production of lactate (1.7 ± 0.38 mmol vs 2.7 ± 0.28 mmol) and Warburg effect than hmoDCs (Fig 2, *A*), and the same tendency was observed after LPS stimulation (2.6 ± 0.18 mmol in mannan-tolDCs vs 3.3 ± 0.34 mmol in hmoDCs). Mannan-tolDCs displayed a significantly higher metabolic rate than hmoDCs under unstimulated conditions, indicating a higher consumption of glucose from the culture medium (Fig 2, *B*). LPS-stimulated hmoDCs significantly increased their metabolic rate with no significant differences compared to LPS-stimulated mannan-tolDCs (Fig 2, *B*). Both unstimulated and LPS-stimulated mannan-tolDCs showed significantly higher mitochondrial mass and mitochondrial membrane potential than unstimulated or LPS-stimulated hmoDCs (Fig 2, *C*), indicating that the glucose metabolic fate in mannan-tolDCs is shifted toward mitochondrial oxidative phosphorylation (OXPHOS). Supporting these data, both unstimulated and LPS-stimulated mannan-tolDCs significantly increased ROS production compared to hmoDCs (Fig 2, *D*). Nicotinamide adenine dinucleotide (NADH) accumulation in LPS-stimulated mannan-tolDCs was significantly reduced compared to hmoDCs, with the same tendency in unstimulated mannan-tolDCs (Fig 2, *D*), suggesting a higher mitochondrial respiratory chain activity. Intracellular ATP production was higher in unstimulated mannan-tolDCs than hmoDCs, without differences after LPS stimulation (Fig 2, *D*). Mannan-tolDCs expressed higher mRNA levels of key metabolic enzymes involved in glycolysis, tricarboxylic acid, and OXPHOS after 4 hours of LPS stimulation, except for lactate dehydrogenase A, than hmoDCs (Fig 2, *E*). Collectively,

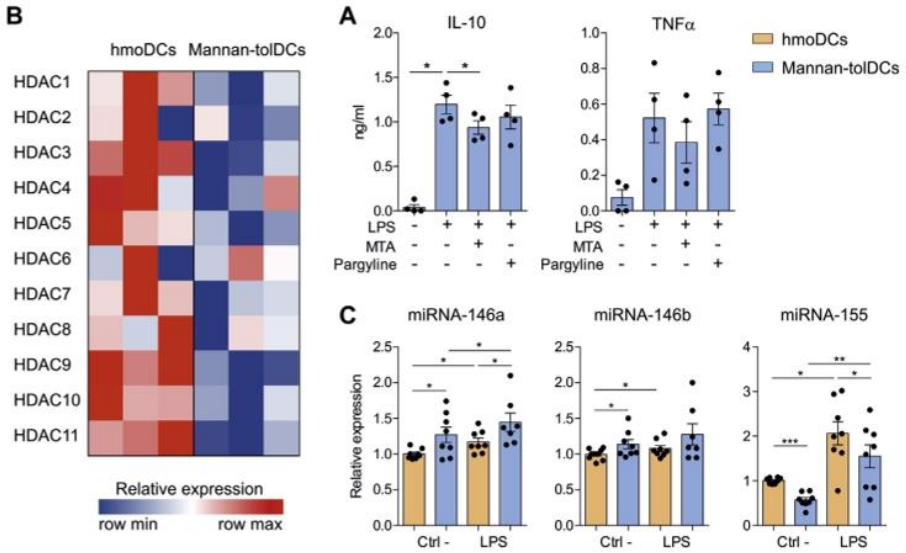


**FIG 4.** Mannan-tolDCs epigenetic reprogramming is associated with tolerogenicity. **A,** Cytokine production by unstimulated or LPS-activated mannan-tolDCs in the presence of MTA or pargyline as inhibitors of histone methyltransferases and demethylases, respectively. **B,** Heat map analysis of mRNA expression of *HDACs* by unstimulated hmoDCs or mannan-tolDCs. **C,** miRNA expression of hmoDCs and mannan-tolDCs stimulated with medium (Ctrl −) or LPS. Results are shown as means ± SEMs of 4 (*A*) and 7-8 (*C*) independent experiments. Paired Student *t* test or Wilcoxon test, $*P < .05$, $**P < .01$, and $***P < .001$.

these data indicate that mannan-tolDCs shifted glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS.

## Mannan-tolDCs epigenetic reprogramming is associated with tolerogenicity

Epigenetics plays an important role in the generation of sustained immunogenic or tolerogenic DCs during monocyte differentiation.[23] To assess the capacity of allergoid–mannan conjugates to imprint epigenetic reprogramming in the generated mannan-tolDCs, we initially studied chromatin status near genes of specific cytokines and tolerogenic molecules. We performed chromatin immunoprecipitation (ChIP) analysis of the active chromatin histone marks H3K4me3 and H3K27ac and of the inhibited chromatin histone mark H3K27me3. The histone modification H3K4me3 (open chromatin and transcription activation) was significantly more associated to *IL10, PDL1*, and *SOCS3* genes in mannan-tolDCs than hmoDCs (Fig 3). Similarly, active H3K27ac was more associated to *PDL1* and *SOCS1* and less associated to *TNFα* in mannan-tolDCs than hmoDCs. In contrast, H3K27me3 (close chromatin and transcription inhibition) was less associated to *IDO* gene in mannan-tolDCs than hmoDCs. These data indicate that generated mannan-tolDCs display epigenetic reprogramming involving specific histone marks associated with the activation of a tolerogenic transcriptional program.

We also performed inhibition experiments using the histone methyltransferase inhibitor 5′-methylthioadenosine (MTA) or the histone demethylase inhibitor pargyline. LPS-stimulated mannan-tolDCs produced significantly less IL-10 in the presence of MTA without significant changes observed in the presence of pargyline (Fig 4, *A*), supporting a role for histone methylation in IL-10 production by mannan-tolDCs. We did not observe significant changes in TNF-α production (Fig 4, *A*), suggesting other alternative mechanisms for this cytokine. In addition, the mRNA levels of the histone deacetylases (*HDACs 1* to *11*) were lower in freshly generated mannan-tolDCs than hmoDCs, suggesting that allergoid–mannan conjugates imprint a global epigenetic reprogramming that ensure histone acetylation and the subsequent open chromatin status within the specifically regulated tolerogenic genes in mannan-tolDCs.

We also wanted to study other mechanisms involved in mannan-tolDCs tolerogenicity. Previous studies have identified microRNA (miRNA)-146 as an anti-inflammatory and miRNA-155 as a proinflammatory miRNA in human DCs.[27,28] miRNA-146a and miRNA-146b expression levels were significantly higher in mannan-tolDCs than hmoDCs, both under unstimulated and LPS-stimulated conditions (Fig 4, *C*). In contrast, miRNA-155 levels were significantly lower in unstimulated mannan-tolDCs than hmoDCs. LPS stimulation increased the expression of miRNA-155 with significantly higher levels observed in hmoDCs than mannan-tolDCs. Mannan-tolDCs were transfected either with locked nucleic acids control or miRNA-146 inhibitor or with control or miRNA-155-5p for miRNA-155 overexpression, then stimulated with LPS or medium for 24 hours without affecting cell viability (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). The effectiveness of the transfections were analyzed by quantifying the miRNA expression levels under the different conditions (Fig E4, *B*). Inhibition of miRNA-146 expression or miRNA-155 overexpression in mannan-tolDCs increased TNF-α production after LPS stimulation, with higher levels observed upon miRNA-155 overexpression (Fig E4, *C*). Changes in IL-10 production by LPS-stimulated mannan-tolDCs were not observed in the assayed conditions (Fig E4, *C*), indicating that miRNA-146 and miRNA-155 contribute to regulate TNF-α but not IL-10 production in LPS-stimulated mannan-tolDCs.

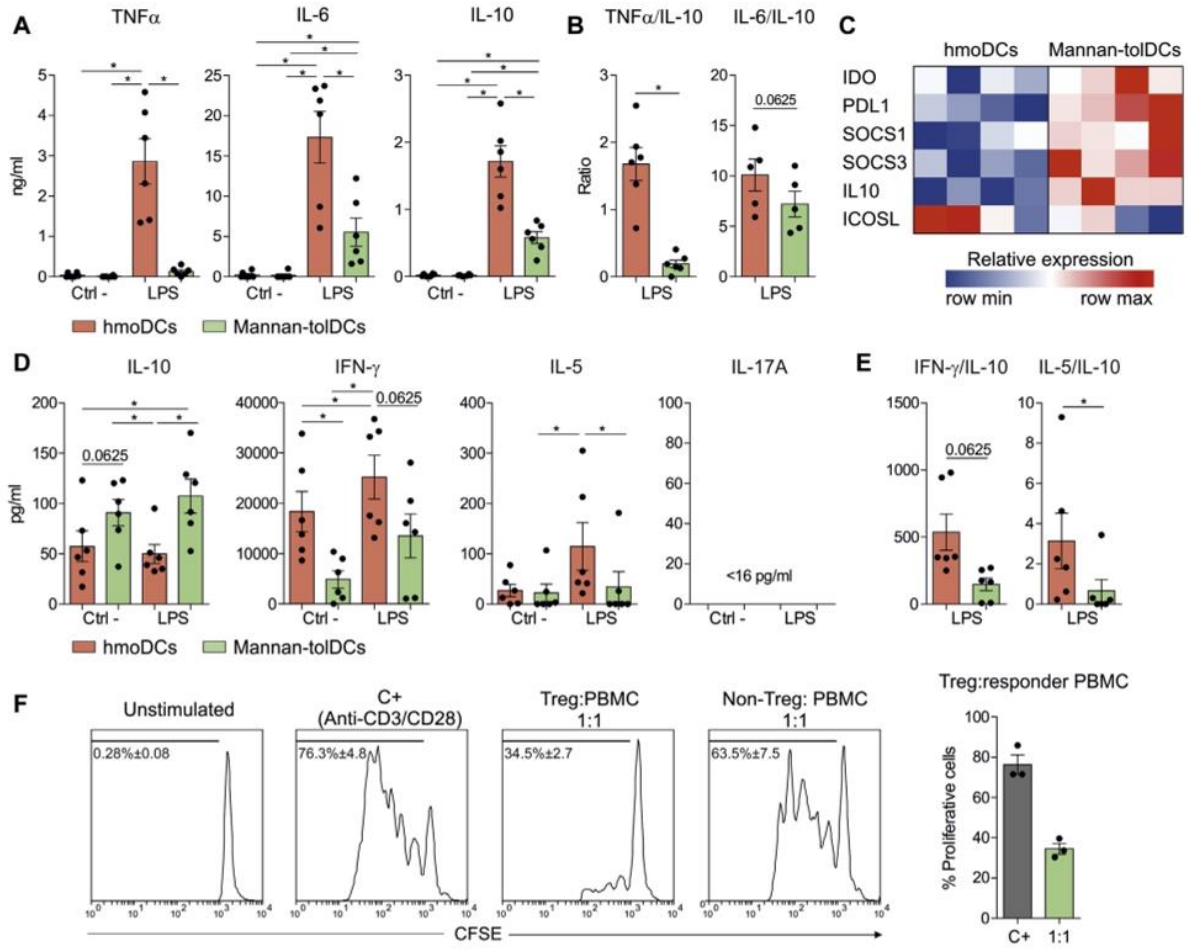


**FIG 5.** Allergoids conjugated to mannan also reprogram the differentiation of monocytes from allergic patients into tolerogenic DCs. **A** and **B,** Cytokine (*A*) or cytokine ratios (*B*) produced after stimulation with medium (Ctrl −) or LPS of conventional hmoDCs or mannan-tolDCs from allergic patients. **C,** Heat map analysis of mRNA expression of tolerogenic molecules by freshly isolated hmoDCs or mannan-tolDCs from allergic patients. **D** and **E,** Cytokines (*D*) or cytokine ratios (*E*) produced by allogeneic naive $CD4^+$ T cells primed by unstimulated or LPS-stimulated hmoDCs or mannan-tolDCs from allergic patients after 5 days. F, Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMCs gated on $CD4^+$ T cells after 5 days of coculture with autologous purified Treg cells and non-Treg cells generated by allogeneic unstimulated mannan-tolDCs (ratio 1:1). The percentage of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition is shown. Results are shown as means ± SEMs of 6 (*A, B, D,* and *E*) and 3 (*F*) independent experiments. Wilcoxon test, $*P < .05$, $**P < .01$, and $***P < .001$.

### Allergoid–mannan conjugates also reprogram the differentiation of monocytes from allergic patients into tolerogenic DCs via epigenetic and metabolic rewiring

To verify that allergoids conjugated to mannan could also reprogram the differentiation of monocytes from allergic patients into tolerogenic DCs, we purified monocytes from allergic patients and generated DCs under different conditions (Fig 1, *A*). LPS-stimulated mannan-tolDCs from allergic patients also produced significantly lower levels of TNF-α and IL-6 than hmoDCs with lower TNF-α/IL-10 and IL-6/IL-10 ratios (Fig 5, *A* and *B*). Mannan-tolDCs from allergic patients showed significantly higher mRNA expression levels of *IDO, PDL1, SOCS1*, *SOCS3,* and *IL10* than hmoDCs (Fig 5, *C*). Unstimulated mannan-tolDCs from allergic patients generated T cells producing higher IL-10 and lower IFN-γ than those generated by hmoDCs (Fig 5, *D*). Remarkably, LPS-stimulated mannan-tolDCs from allergic patients generated T cells producing higher levels of IL-10 and lower levels of IFN-γ and IL-5 than T cells generated by hmoDCs (Fig 5, *D*). The IFN-γ/IL-10 and IL-5/IL-10 ratios were also significantly lower in LPS-stimulated mannan-tolDCs cocultures (Fig 5, *E*). Purified Treg cells ($CD4^+CD25^+CD127^-$) generated by mannan-tolDCs from allergic patients but not $CD4^+CD25^-CD127^-$ non-Treg cells inhibited the proliferation of autologous PBMCs in a dose-dependent manner (Fig 5, *F,* and see Fig E3, *C,* in this article's Online Repository at www.jacionline.org). Interestingly, both mannan-tolDCs unstimulated and loaded with native grass

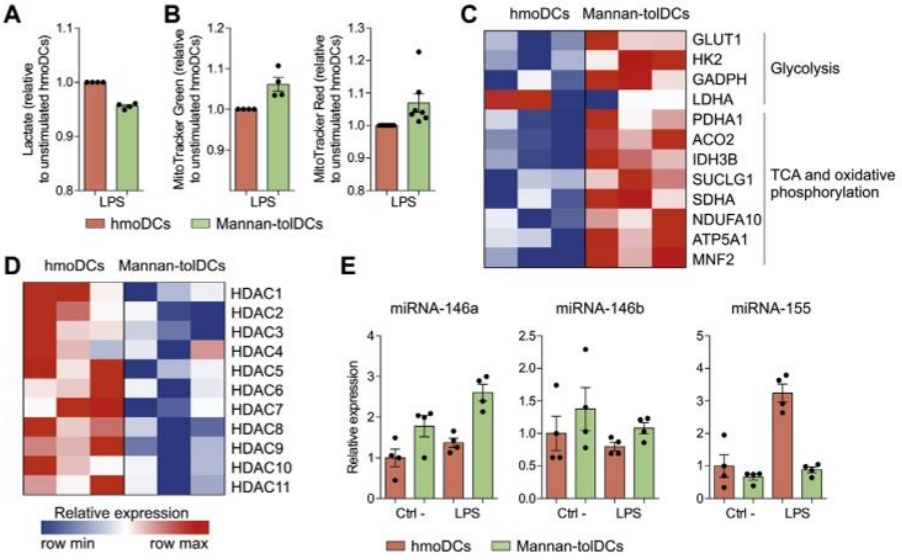


**FIG 6.** Mannan-tolDCs from allergic patients also display epigenetic and metabolic rewiring. **A,** Lactate content in cell-free supernatants from LPS-stimulated hmoDCs and mannan-tolDCs from allergic patients after 18 hours relative to LPS-stimulated hmoDCs. **B,** Fluorescence intensity of cells from allergic patients stained with MitoTracker Green (mitochondrial mass) or MitoTracker Red (mitochondrial membrane potential) relative to LPS-stimulated hmoDCs. **C,** Heat map of mRNA expression levels of metabolic genes in hmoDCs and mannan-tolDCs from allergic patients treated with LPS for 4 hours. **D,** Heat map analysis of mRNA expression of *HDACs* by unstimulated hmoDCs or mannan-tolDCs from allergic patients. **E,** miRNA expression of hmoDCs and mannan-tolDCs from allergic patients stimulated with medium or LPS. Results are shown as means ± SEMs of 4 (*A*), 4-6 (*B*), and 4 (*E*) independent experiments.

pollen allergens increase in the generation of FOXP3$^+$ Treg cells compared to conventional hmoDCs, indicating that mannan-tolDCs from allergic patients are able to induce and expand allergen-specific Treg cells (see Fig E5 in this article's Online Repository at www.jacionline.org).

Metabolically, LPS-stimulated mannan-tolDCs from allergic patients also displayed lower production of lactate (Warburg effect) and higher mitochondrial mass and mitochondrial membrane potential than allergic hmoDCs (Fig 6, *A* and *B*). Mannan-tolDCs from allergic patients showed increased mRNA expression levels of glycolytic, tricarboxylic acid cycle, and OXPHOS genes than hmoDCs (Fig 6, *C*). The mRNA expression levels for *HDACs 1* to *11* were also lower in mannan-tolDCs than hmoDCs from allergic patients (Fig 6, *D*). Finally, the miRNA expression pattern in mannan-tolDCs from allergic patients was the same as that described in nonatopic donors (Fig 6, *E*). Collectively, these data demonstrate that allergoids conjugated to mannan are also able to differentiate monocytes from allergic patients into mannan-tolDCs showing tolerogenic properties via metabolic and epigenetic reprogramming.

## DISCUSSION

In this study, we show for the first time that next-generation AIT vaccines based on allergoids conjugated to mannan reprogram monocyte differentiation and generate tolerogenic DCs via epigenetic and metabolic rewiring in both nonatopic donors and allergic patients. Mechanistically, generated mannan-tolDCs shift glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS, a metabolic hallmark of tolerogenic DCs.[24] Allergoid–mannan conjugates imprint epigenetic reprogramming in the generated mannan-tolDCs involving specific histone marks, thus ensuring sustained open chromatin status within the specifically regulated tolerogenic genes during monocyte differentiation, leading to tolerogenic DCs with stable phenotypes even upon proinflammatory stimulation. In addition, mannan-tolDCs significantly increase the expression of the anti-inflammatory miRNA-146a/b whereas they decrease proinflammatory miRNA-155, which also contributes to the imprinted tolerogenic features. Overall, we uncovered unprecedented molecular mechanisms by which allergoid–mannan conjugates might well also restore tolerance to allergens during AIT.

We previously showed that allergoid–mannan conjugates target DCs via mannose receptor and DC-SIGN, enhancing allergen uptake, increasing IL-10 production and PD-L1 expression, and promoting the generation of functional allergen-specific of FOXP3$^+$ Treg cells both *in vitro* and *in vivo,* which was impaired by the conventional adjuvant alum.[12,14,16] However, whether allergoid–mannan conjugates could also regulate human monocyte differentiation into DCs and the underlying molecular mechanisms remained completely unknown. Vaccine administration sites are usual places of monocyte recruitment,[21,22] and there is compelling experimental evidence showing that monocyte differentiation into inflammatory or tolerogenic DCs is highly conditioned by the signals encountered in the local tissue microenvironment.[18-20,29] For example, endogenous and synthetic molecules such as IL-10, TGF-β, glucocorticoids, rapamycin, minocycline, ethyl pyruvate, or vitamin $D_3$, among others, promote the *in vitro* generation of

human monocyte–derived tolerogenic DCs.[19,20,30-32] Some of them, such as dexamethasone, minocycline, ethyl pyruvate, or vitamin $D_3$, also induce tolerogenic DCs in mice *in vivo*.[20,33] Here, we demonstrate that allergoid–mannan conjugates can also drive monocyte differentiation into tolerogenic DCs in both nonatopic and allergic subjects. The generated mannan-tolDCs significantly increase the expression of classical tolerogenic molecules and polarize functional $FOXP3^+$ Treg cells. Remarkably, mannan-tolDCs from nonatopic subjects and allergic donors displayed a stable tolerogenic phenotype under inflammatory conditions, as demonstrated by their lower production of proinflammatory cytokines and significantly lower TNF-α/IL-10 ratio compared to conventional DCs upon LPS stimulation. Supporting these data, LPS-stimulated mannan-tolDCs also induced higher numbers of IL-10–producing Treg cells with concomitant lower generation of effector $T_H1$ and $T_H2$ cells than conventional DCs.

These results are aligned with the observed higher expression of *SOCS1* and *SOCS3* genes in mannan-tolDCs, which are among the most relevant cytokine signaling suppressor molecules.[34,35] Similarly, PD-L1 and indoleamine 2,3-dioxygenase (IDO) have been previously linked to the capacity of tolerogenic DCs to induce functional $FOXP3^+$ Treg cells.[14,36-39] There might be some evidence suggesting the potential mechanisms by which these processes might also occur *in vivo:* (1) allergoid–mannan conjugates induce high levels of IL-10 but also IL-6 by already differentiated DCs,[14] which could act as an initial proinflammatory signal enhancing monocyte recruitment; (2) allergoid–mannan conjugates are hypoallergenic, but their residual allergenicity[14] might well induce the signals and cytokine milieu for monocyte recruitment and differentiation; (3) allergoid–mannan conjugates induce spleen Treg cells, which are impaired by oxidation of mannan or by the presence of alum at the injection site;[14,16] (4) allergoid–mannan conjugates are efficiently captured by human monocyte–derived DCs,[14] which could then migrate to draining lymph nodes and transfer the antigens to other cells migrating from the injection site by different mechanisms, thus facilitating additional contact with the vaccine; (5) allergoid–mannan conjugates, which are structurally very stable, are also larger in size than allergoids without mannan, resulting in a lower diffusion rate.[13] While all these structural features may support a persistent presence of the allergoid–mannan conjugates at the injection site, infiltrating myeloid cells will be able to take up these conjugates even if a low concentration exists because this uptake is favored by a receptor-mediated mechanism.[14]

To gain further insight into the molecular mechanisms underlying the stable tolerogenic phenotype induced in mannan-tolDCs, we sought to investigate potential metabolic and epigenetic reprogramming imprinted by allergoid–mannan conjugates during monocyte differentiation. Different studies have shown that the regulation of the functional properties of human DCs is highly connected with metabolic reprogramming.[25,26] Our data revealed that mannan-tolDCs from nonatopic and allergic patients shift glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS under steady-state conditions and upon LPS stimulation, indicating a metabolic rewiring that has been previously associated with tolerogenic human DCs.[24-26] Previous studies reported that LPS-stimulated tolerogenic hmoDCs highly express genes involved in energetic metabolism, including genes related to glycolytic and mitochondrial catabolic pathways.[24] We observed an increased expression of tricarboxylic acid cycle and OXPHOS genes in mannan-tolDCs from nonatopic controls and allergic patients. It has been described that many OXPHOS genes are highly expressed in tolerogenic hmoDCs, indicating the potential for a higher mitochondrial activity, with higher production of ROS than immature and mature hmoDCs.[24-26,40] In agreement with our data, it has also been demonstrated that tolerogenic hmoDCs display an increased expression of most of the glycolytic genes relative to mature hmoDCs.[24] We only observed lower gene expression for lactate dehydrogenase A enzyme in mannan-tolDCs, which is also aligned with the detected reduction of lactate production. We previously showed that allergoid–mannan conjugates directly activate glycolysis accompanied with ROS production in hmoDCs, which was essential for $FOXP3^+$ Treg generation and impaired by alum.[16] Herein, we show that mannan-tolDCs also display enhanced glycolysis with a clear shift toward OXPHOS and ROS production, suggesting that although certain levels of lactate are needed, a mitochondrial shift and ROS production are indispensable to generating tolerogenic DCs during monocyte differentiation. Taken together, our data indicate that allergoid–mannan conjugates not only imprint tolerogenic features in already differentiated DCs but also reprogram monocyte differentiation into mannan-tolDCs by imprinting a glucose metabolic status typically associated with tolerogenic DCs.

Epigenetics plays a crucial role in regulating gene expression during monocyte differentiation into DCs and in the generation of stable transcriptional programs in immunogenic versus tolerogenic DCs.[20] Our ChIP data revealed that the permissive chromatin marks H3K4me3 and H3K27ac are more enriched near genes of tolerogenic molecules such as *IL10, PDL1, SOCS1*, and *SOCS3* in mannan-tolDCs than conventional DCs, which might well justify the stable tolerogenic profile observed for mannan-tolDCs. Accordingly, the inhibition of histone methylation with MTA in LPS-stimulated mannan-tolDCs significantly reduced the production of IL-10, indicating that its increased production might be regulated by histone methylation, as shown with the higher H3K4me3. We did not observe changes in TNF-α production, suggesting that other alternative mechanisms may be involved in the regulation of this proinflammatory cytokine.

Mannan-tolDCs from both nonatopic donors and allergic patients display a lower expression of all the *HDAC* quantified isoforms (HDACs 1 to 11) compared to conventional DC counterparts. It has been previously shown that HDAC activity is a crucial driver of allergic inflammation and that HDAC inhibition reduces allergic inflammation; it has thus been proposed as a potential anti-inflammatory strategy for the treatment of allergic airway diseases.[41-43] HDAC inhibitors also increase the number and function of naturally occurring Treg cells, regulating several DC functions through the induction of IDO,[44] an enzyme that is upregulated at the mRNA level and that is less associated with the inhibitory histone mark H3K27me3 on mannan-tolDCs than conventional DCs. Along the same lines, it has been previously reported that the nuclear receptor corepressor 1 is a master negative regulator of tolerogenesis in murine conventional DCs and hematopoietic stem cell precursors by mechanisms partially depending on the recruitment of HDACs to promote compact close chromatin within tolerogenic genes.[45,46] Overall, our data suggest that allergoid–mannan conjugates imprint a global epigenetic

reprogramming that ensure histone acetylation and subsequent open chromatin status within the specifically regulated tolerogenic genes in mannan-tolDCs, thus favoring a stable tolerogenic DC phenotype. miRNAs also play a crucial role in the generation of human tolerogenic DCs during monocyte differentiation.[47,48] The upregulation of miRNA-155 during inflammatory processes has been correlated with the hyperactivation of myeloid cells, while inhibition of miRNA-155 increases the expression of suppressor of cytokine signaling (SOCS) 3.[49] In line with these data, the generated mannan-tolDCs from nonatopic and allergic patients display lower expression of miRNA-155 and higher SOCS3 expression than conventional DCs. In addition, anti-inflammatory miRNA-146, especially miRNA-146a, is significantly increased in nonatopic and allergic mannan-tolDCs, indicating that allergoid–mannan conjugates induce a miRNA signature in mannan-tolDCs that contributes to reinforce the imprinted transcriptional tolerogenic program during monocyte differentiation.

In summary, we uncovered novel molecular mechanisms by which allergoid–mannan vaccines might contribute to restoring tolerance to allergens during AIT. Our findings showing that allergoid–mannan conjugates reprogram monocytes from nonatopic donors and allergic patients into mannan-tolDCs via epigenetic and metabolic reprogramming significantly enhance our knowledge regarding the mode of action of AIT vaccines aimed at inducing tolerance to allergens. Our results elucidating novel epigenetic and metabolic reprogramming mechanisms induced by allergoid–mannan conjugates during monocyte differentiation could help pave the way for the identification of novel biomarkers for the management of allergic patients undergoing this AIT and for the design of alternative antigen-specific DC-based strategies for allergy and other inflammatory diseases.

**Clinical implications: We uncovered innate immune mechanisms contributing to allergen tolerance induction that might help identify novel allergen-specific immunotherapy biomarkers and to design alternative dendritic cell–based strategies for allergy and other inflammatory diseases.**

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## METHODS

### Material, media, and reagents

For cell cultures, we used RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL normocin (InvivoGen, San Diego, Calif), 50 μg/mL penicillin–streptomycin, 1% nonessential amino acids, 1% MEM vitamins, and 1 mmol/L sodium pyruvate (Life Technologies, Carlsbad, Calif). LPS (0127:B8) and inhibitors for histone methyltransferase MTA and histone demethylase (pargyline) (Sigma-Aldrich, St Louis, Mo). Allergoid–mannan conjugates (Inmunotek, Madrid, Spain) were performed by conjugation of grass pollen *Phleum pratense* allergens with mannan from *Saccharomyces cerevisiae* through the lysine residues from its mannoprotein in a single step. Briefly, glutaraldehyde (25 mmol/L) was added to a solution containing the mixture of allergen and mannan (1:0.5 wt/wt) in PBS for 6 hours at 4°C with continuous stirring. The reaction was stopped with glycine (1.25 mol/L), followed by tangential flow filtration with distilled water (membrane cutoff, 100 kDa). Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan were recovered in the concentrated retained fraction (>100 kDa). Mannan (Sigma-Aldrich, 50 μg/mL) was used to differentiate monocytes into dendritic cells (DCs) for comparing experiments.

### HmoDC generation and naive $CD4^+$ T-cell purification

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of nonatopic controls or allergic patients by Ficoll density gradient centrifugation (800 × *g*, 20 minutes). Immature hmoDCs were generated from blood monocytes obtained from total PBMCs using anti-CD14 microbeads (Miltenyi Biotec, San Diego, Calif) and cultured for 6 days with RPMI 1640 medium containing 100 ng/mL of IL-4 and granulocyte–macrophage colony-stimulating factor (PeproTeck, Rocky Hill, NJ). To generate mannan-tolDCs, allergoid–mannan conjugates (50 μg/mL) were added on days 0 and 4 of differentiation. To generate allergoid DCs, mannan-free glutaraldehyde-polymerized grass pollen allergoids (50 μg/mL) were added on days 0 and 4 of the differentiation. 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 from buffy coats of nonatopic controls using the Naive $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer's protocol. In all the cases, viable cells were counted using trypan blue staining and an optical microscope, and analyzed by propidium iodide by flow cytometry.

### Cell cultures

hmoDCs, mannan-tolDCs, or allergoid DCs from nonatopic controls or allergic patients were treated with medium or LPS (100 ng/mL) for 18 hours. Cell pellets were used to analyze their phenotype by real-time quantitative PCR (qPCR) and cell-free supernatants to quantify IL-6, TNF-α, and IL-10 by ELISA. For coculture experiments, hmoDCs or mannan-tolDCs treated with medium (Ctrl −) or LPS (100 ng/mL) were cocultured with purified allogeneic naive $CD4^+$ T cells (DC/T-cell ratio of 1:5) for 5 days. IL-17A, IFN-γ, IL-5, and IL-10 were quantified in cell-free supernatants by ELISA. To analyze the induction of allergen-specific Treg cells, hmoDCs or mannan-tolDCs from allergic patients were treated with medium or native grass pollen allergens (50 μg/mL) for 18 hours, then cocultured with autologous peripheral blood lymphocytes at a DC/peripheral blood lymphocyte ratio of 1:5 to analyze $FOXP3^+$ Treg cells.

For inhibition experiments, mannan-tolDCs were preincubated for 1 hour with MTA (50 μmol) or pargyline (5 μmol) (or their vehicle control, dimethyl sulfoxide) before activation. Then cells were stimulated with LPS for 18 hours in the presence of the corresponding inhibitors to quantify TNF-α and IL-10 by ELISA. Cell viability was analyzed in all cases by trypan blue exclusion with a light microscope.

### Cytokine quantification

Concentrations of IL-6, TNF-α, IL-10, IFN-γ, and IL-5 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, Calif). IL-17A levels were quantified by quantikine Elisa Kit (R&D Systems, Minneapolis, Minn). In all cases, manufacturer's instructions were followed with minor modifications.

### RNA extraction, complementary DNA synthesis, and qPCR

RNA was isolated from collected cells with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For mRNA analysis, complementary DNA was generated with a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). qPCR was performed on complementary DNA by using FastStart Universal SYBR Green Master (Rox; Roche, Basel, Switzerland). The sequences of the pair primers we used are listed in Table E1. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems, Thermo Fisher Scientific, Waltham, Mass). Data were normalized to EF1α and displayed as $2^{-\Delta Ct}$ values multiplied by $10^4$, with change in cycle threshold (ΔCt) defined as the difference between the Ct value for the gene of interest and EF1α. For miRNA analysis, RNA isolation QIAzol (Qiagen) and Total RNAzol Out Mini Kit (A&A Biotechnology, Gdynia, Poland) were used according to the manufacturer's instructions. To analyze miRNA expression, TaqMan MicroRNA Assays (Life Technologies) and 5×HOT FIREPol Probe qPCR Mix Plus (Rox; Solis BioDyne, Tartu, Estonia) were used according to the manufacturer's protocols. Each PCR reaction was performed using a ViiA 7 real-time PCR system (Life Technologies). For normalization, let-7a and ΔΔCt calculation were used.

### Flow cytometry

Flow cytometric monoclonal antibodies were purchased from BioLegend (San Diego, Calif): human anti-FOXP3–Alexa Fluor 488, anti-CD127–phycoerythrin, anti-CD25–allophycocyanin, and anti-CD4–peridinin chlorophyll protein complex [PerCP]. For analysis of FOXP3 expression in human T cells primed with DCs, cells were first subjected to surface staining with anti-human CD4-PerCP, CD127–phycoerythrin, and CD25–allophycocyanin antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3–Alexa Fluor 488 according to the manufacturer's recommendations. The corresponding isotype controls were included in each staining. Flow cytometry analysis was performed with a FACSCalibur cytometer (Becton Dickinson, San Diego, Calif) in the cytometry and fluorescence microscopy unit at Complutense University of Madrid.

### Treg cell–suppression assay

$CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells induced by unstimulated allogeneic mannan-tolDCs were purified by cell sorting the $CD4^+CD25^{high}CD127^-$ population and mixing with carboxyfluorescein succinimidyl ester (CFSE)-labeled autologous PBMCs (responder cells) at a 1:1 ratio and stimulated with plate-bound anti-human CD3 antibody (1 mg/mL, clone OKT3; eBioscience, San Diego, Calif) and soluble anti-human CD28 (1 mg/mL, clone CD28.6; eBioscience) for 5 days. For control purposes, CFSE-labeled PBMCs were cultured alone with or without stimulation, and non-Treg cells ($CD4^+CD25^-CD127^-$) were also tested. Proliferation of $CD4^+$ T cells was determined by using CFSE dilution with flow cytometry.

### Metabolic studies

The Warburg effect in stimulated DC cultures was determined photometrically 18 hours after stimulation by quantifying the OD at 570 nm and calculating the Warburg effect as 1/OD 570 nm normalized to the unstimulated cells. Lactate concentrations in culture supernatants were determined 18 hours after stimulation by using the colorimetric L-Lactate Assay kit (Abcam, Cambridge, UK) according to the manufacturer's recommendations. Glucose concentrations in culture supernatants were determined 18 hours after stimulation by using the Glucose (GO) Assay Kit

(Sigma-Aldrich). The metabolic rate was derived mathematically in the percentage of medium without DCs (glucose concentration in RPMI 1640 = 2 mg/mL). To measure mitochondrial mass and mitochondrial membrane potential, MitoTracker Green FM (25 nmol) or MitoTracker Red CMXRos (250 nmol) were used, respectively (Thermo Fisher Scientific). Arbitrary fluorescence values were obtained in a FLUOstar Omega microplate reader (Ex/Em = 490/516 nm for Mitotracker Green and Ex/Em = 579/599 nm for Mitotracker Red CMXRos). Reactive oxygen species (ROS) were measured by the DCDFA Cellular ROS Detection Assay Kit (Abcam) following the manufacturer's instructions. Briefly, total ROS levels were measured using the cell-permeant reagent 2′,7′-dichlorofluorescin diacetate (DCDFA). Cells were stained with DCDFA for 30 minutes at 37°C after 18 hours of treatment with the corresponding stimuli. Fluorescence was measured in a FLUOstar Omega microplate reader (Ex/Em = 485/535 nm). Total NADH concentrations in lysates of DCs were determined by using the NAD/NADH Assay Kit (Abcam) according to the manufacturers' recommendations. For NADH measures, well volume was 160 μL. ATP concentrations in lysates of hmoDCs were determined with the ATP Determination Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions.

## Chromatin immunoprecipitation

For chromatin immunoprecipitation (ChIP) analysis, hmoDCs and mannan-tolDCs were fixed after differentiation. Cells were subjected to lysis and sonication using the Bioruptor UCD-200 sonicator (Diagenode, Liege, Belgium) to obtain chromatin fragments of 200 to 400 bp. Sheared chromatin was immunoprecipitated with 2 μL of anti-H3K4me3 antibody (EMD Millipore, Billerica, Mass, catalog no. 07-473), 2 μL of anti-H3K27me3 antibody (Millipore, 07-449), 2 μL of anti-H3K27ac antibody (Abcam, ab4729) or 2 μL of anti-IgG antibody (Millipore, 12-370) and 3 μL of Dynabead M-280 magnetic beads (sheep anti-mouse IgG, sheep anti-rabbit IgG, Life Technologies) using the SX-8G IP-Star Automated System (Diagenode). ChIP samples were de–cross-linked at 65°C for 4 hours. After incubation, the samples were treated with 0.2 mg/mL RNaseA (RNase Cocktail Enzyme Mix; Ambion, Life Technologies) and 0.4 mg/mL proteinase K (Thermo Fisher Scientific). DNA was purified with the DNA Clean Kit & Concentrator TM-5 (Zymo Research, Irvine, Calif) according to the manufacturer's protocol. qPCR was performed on the purified ChIP and INPUT DNAs by using FastStart Universal SYBR Green Master. Sequences of the pair primers we used are listed Table E1. Data from H3K4me3 and H3K27ac marks were normalized to B2M positive control, and data from H3K27me3 mark were normalized to MYOD1 negative control.

## Transfection experiments

Transfection with NickFect71 was used for Fig 4, *D,* and Fig E2, and was performed as previously described.[E1] Briefly, NickFect71 was incubated with miRNA mimic or lock nucleic acid (LNA) as inhibitor at a 17:1 CPP (cell-penetrating peptide):miRNA molar ratio in MQ water in one tenth of the final treatment volume at room temperature for 1 hour to form CPP:miRNA complexes, then mixed with media and added to the cells. After 24 hours, mannan-tolDCs were stimulated with 100 ng/mL of LPS for 24 hours or left unstimulated. Transfections in DCs were performed at 100 nmol of miRNA mimic negative control, miR-155-5p (Life Technologies), and miRCURY LNA inhibitors (microRNA Power Inhibitor hsa-miR-146 and Negative Control A, Exiqon, Vedbaek, Denmark). Cells were collected in QIAzol and kept at −80°C until RNA isolation.

## ELISA of serum-specific IgE from allergic patients to native grass pollen allergens

Microtiter plates (Corning, Corning, NY) were coated with 10 μg of N in 100 μL of PBS overnight at 4°C. Plates were washed 3 times with PBS and 0.1% vol/vol Tween 20 and blocked for 1 hour with PBS, 0.1% vol/vol Tween 20, and 3% wt/vol dehydrated milk. Then plates were incubated for 2 hours with individual serum from patients with well-defined grass pollen allergy diluted in PBS, 0.1% vol/vol Tween 20, and 3% wt/vol dehydrated milk. After 4 washes, bound IgE antibodies were detected by incubating for 2 hours with goat anti-human IgE-biotin (1:2000 diluted) (Thermo Fisher Scientific), followed by 1 hour with horseradish peroxidase–coupled streptavidin (diluted 1:500). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% H2O2 and 0.63 mg/mL *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0), and the reaction was stopped with 3 N H2SO4. OD was measured at 492 nm in an ELISA reader.

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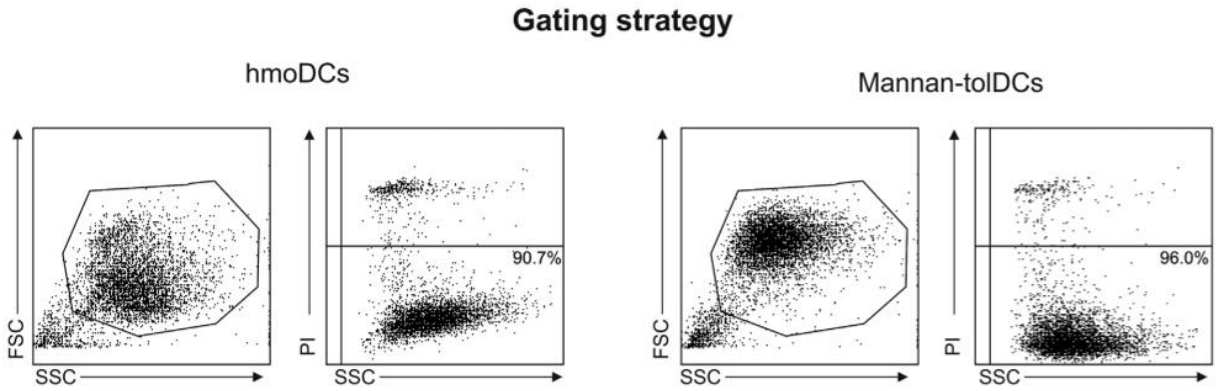


**FIG E1.** The presence of allergoid–mannan conjugates during monocyte differentiation did not affect cell viability. Gating strategy for propidium iodide (PI) labeling analysis with representative histograms of conventional hmoDCs and mannan-tolDCs viability.

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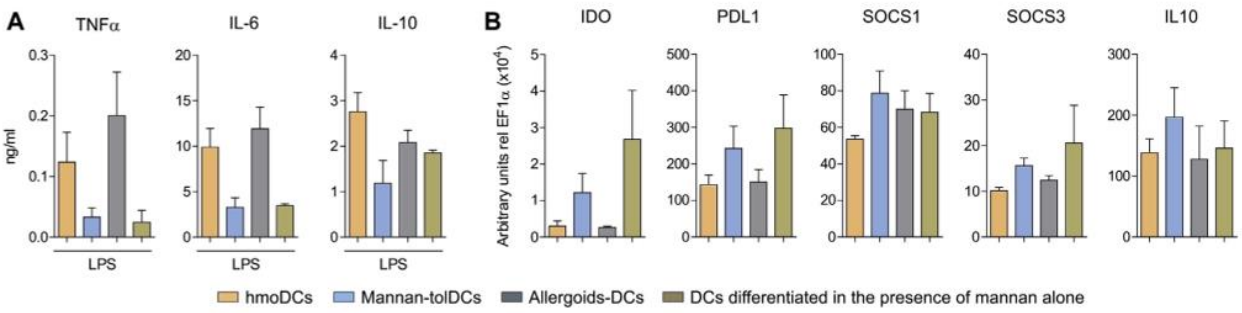


**FIG E2.** The presence of mannan in the conjugate structure is essential for the induction of tolerogenic DCs. **A,** Cytokines produced after no stimulation (Ctrl −) or LPS stimulation of hmoDCs, mannan-tolDCs, or hmoDCs differentiated in the presence of allergoids (allergoid DCs) or hmoDCs differentiated in the presence of mannan alone (n = 3-4). **B,** mRNA expression levels of genes in freshly isolated hmoDCs, mannan-tolDCs, allergoid DCs, and DCs differentiated in the presence of mannan alone. Arbitrary units (AU) are $2^{-\Delta Ct}$ values multiplied by $10^4$, with ΔCt defined as the difference between the Ct value for the gene of interest and EF1α (n = 3).

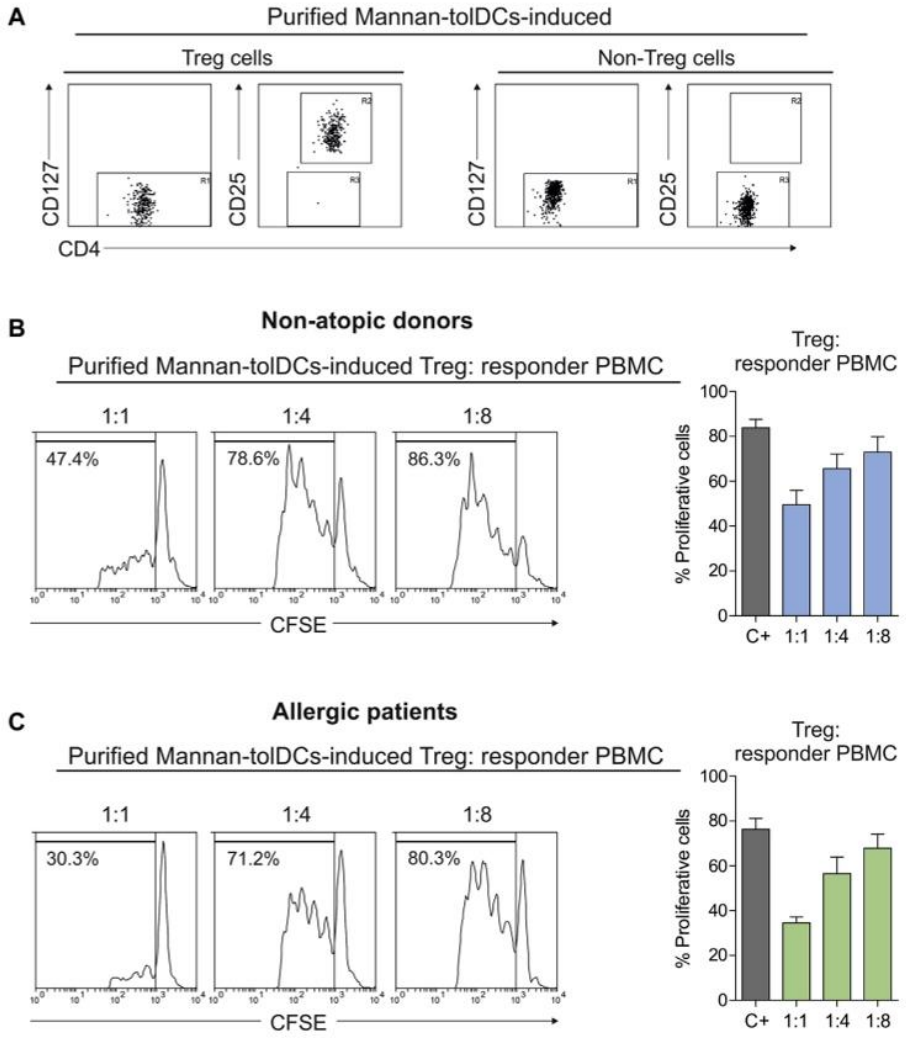


**FIG E3.** Suppression capacity of Treg cells induced by allogeneic mannan-tolDCs from nonatopic and allergic subjects. **A,** Representative dot plots of the purification of Treg cells by sorting. **B** and **C,** Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMCs gated on $CD4^+$ cells after 5 days of coculture with autologous purified Treg cells induced by allogeneic mannan-tolDCs from nonatopic donors (*B*) or mannan-tolDCs from allergic patients (*C*). The percentages of proliferating responder PBMCs stimulated with anti-CD3 and anti-CD28 for each condition at different Treg cell/responder PBMC ratios are shown (n = 3).

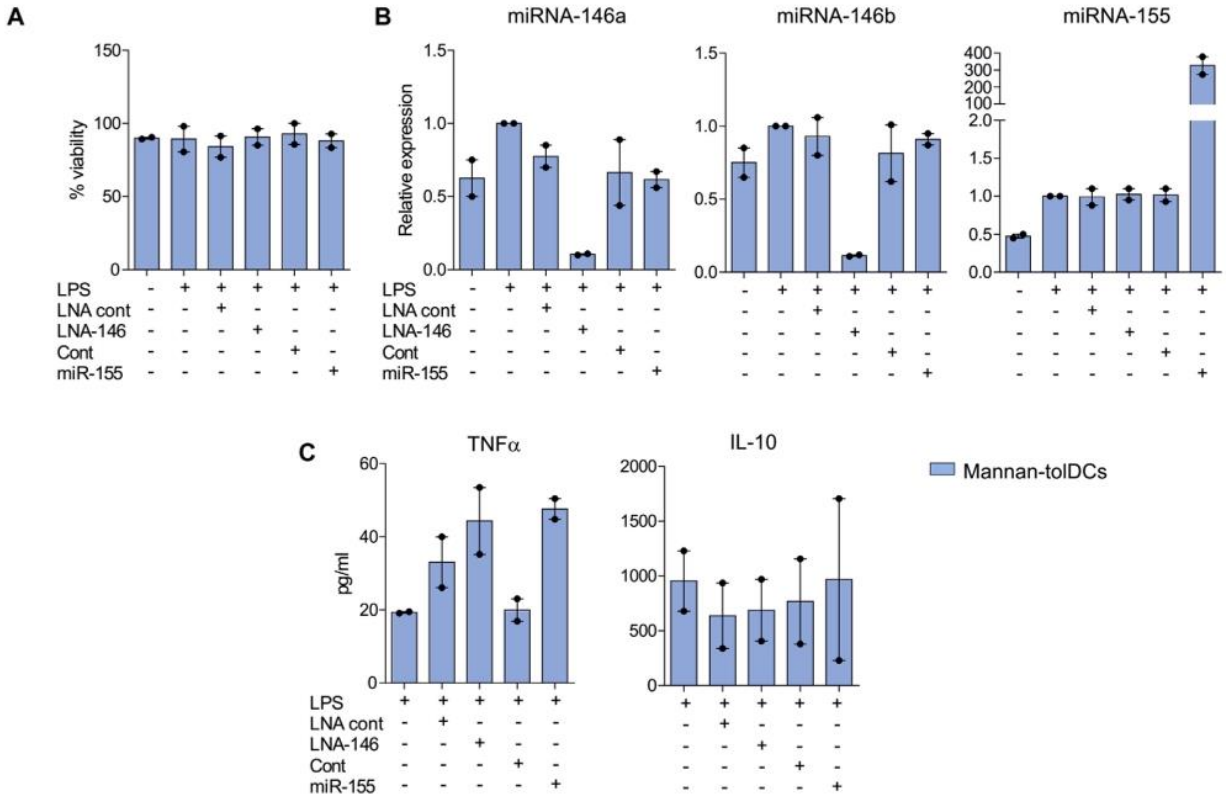


**FIG E4.** Inhibition of miRNA-146 and overexpression of miRNA-155 in mannan-tolDCs. **A,** Viability of unstimulated or LPS-stimulated mannan-tolDCs after transfection with miRNA-146 inhibitor (LNA-146), miRNA-155 or the corresponding vehicle controls (LNA cont or cont). **B,** Expression of indicated miRNAs in the transfected cells. **C,** TNF-α and IL-10 production by mannan-tolDCs transfected with miRNA-146 inhibitor (LNA-146), miRNA-155, or the corresponding vehicle controls (LNA cont or cont) and stimulated with LPS for 24 hours. Results are shown as means ± SEMs of 2 independent experiments.

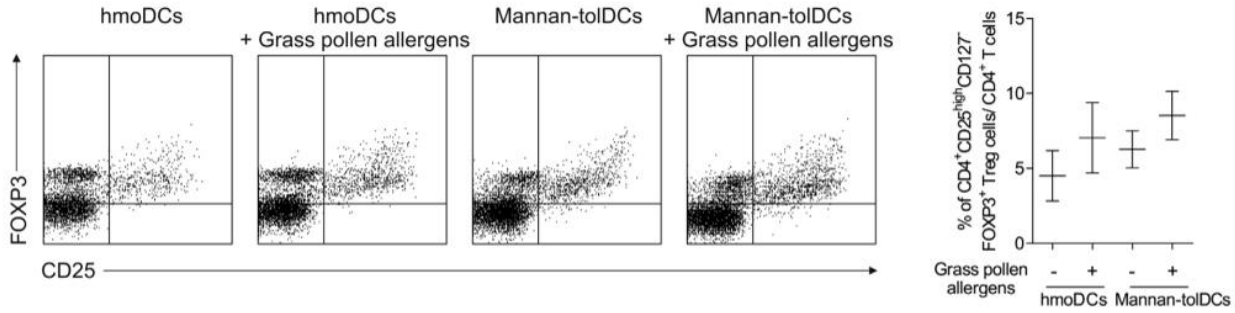


**FIG E5.** Induction of allergen-specific Treg cells. **A,** Percentage and representative dot plots of induced $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells by autologous unstimulated or stimulated with grass pollen allergens mannan-tolDCs from allergic patients after 5 days of coculture with peripheral blood lymphocytes (gating in lymphocytes) (n = 2).

**TABLE E1.** PCR primers

| Gene | Primer sequences | |
|---|---|---|
| | Forward | Reverse |
| *PDL1* | AAGATGAGGATATTTGCTGTCTTTATATTC | GTCCTTGGGAACCGTGACAGT |
| *IDO* | AGAAGTGGGCTTTGCTCTGC | TGGCAAGACCTTACGGACATCTC |
| *SOCS1* | CCCTGGTTGTTGTAGCAGCTT | CAACCCCTGGTTTGTGCAA |
| *SOCS3* | CCTCAGCATCTCTGTCGGAAGA | GCATCGTACTGGTCCAGGAACT |
| *ICOSL* | CTCCGCCCGCACCAT | CTACCATCGCTCTGACTTCCTTCT |
| *IL10* | GTGATGCCCCAAGCTGAGA | CACGGCCTTGCTCTTGTTTT |
| *EF1α* | CTGAACCATCCAGGCCAAAT | GCCGTGTGGCAATCCAAT |
| *GLUT1* | GGCTTCTCCAACTGGACCTC | CCGGAAGCGATCTCATCGAA |
| *HK2* | TTCGCACTGAGTTTGACCAG | TCACCAGGATAAGCCTCACC |
| *GADPH* | CTGCACCACCAACTGCTTAGC | TCATGTTCTGGAGAGCCCCG |
| *LDHA* | ATGGCAACTCTAAAGGATCAGC | CCAACCCCAACAACTGTAATCT |
| *PDHA1* | ATGGAATGGGAACGTCTGTTG | CCTCTCGGACGCACAGGATA |
| *ACO2* | AATGGATGTACTCGTTGGGC | ACAGCCTACTGGTGACTCGG |
| *IDH3B* | CTGATGCACGCCGTCAAG | GCCATATTCTGCACCTCACTCA |
| *SUCLG1* | CCTACACAGCTTCTCGGCAA | CTGTGAAAGGTGCCCTGTTTG |
| *SDHA* | CGAACGTCTTCAGGTGCTTT | AAGAACATCGGAACTGCGAC |
| *NDUFA10* | ACAGAACGCAGCAGAGTGATA | GGAAAGTGCTTGAAGCCTAGTT |
| *ATP5A1* | AAGACACGCCCAGTTTCTTC | TTTGGGTTCATCTTTCATTGC |
| *MNF2* | TCTGGGACCTTTGCTCATCT | CAACCAACCGGCTTTATTCC |
| *HDAC1* | ACTACGACGGGGATGTTGGA | CAG CAT TGG CTT TGT GAG GG |
| *HDAC2* | TCTGCTACTACTACGACGGTGA | TCATTTCTTCGGCAGTGGCT |
| *HDAC3* | ATCTCTGCAAGGAGCAACCC | AGCCAGAGGCCTCAAACTTC |
| *HDAC4* | CCACCTCACTCCCTACCTGA | CCCAGGCCTGTGACGAG |
| *HDAC5* | TCCCGTCCGTCTGTCTGTTA | GACCTGACATCCCATCCGAC |
| *HDAC6* | CTGGCGGAGTGGAAGAACC | GGGGAACGGCTCCCTTTTTA |
| *HDAC7* | CTCGGAGGCTGACAGTGACC | GAGGGGTCCAGGAGGAGAAT |
| *HDAC8* | CAGAAGGTCAGCCAAGAGGG | GGCAGTCATAACCTAGCCCA |
| *HDAC9* | GACGTGTGGTGTTGGCTCTA | GTGGCTCCAGCTCATTTCCT |
| *HDAC10* | TACCATGAGGACATGACGGC | GGGATACATACTCTGGGCTGTG |
| *HDAC11* | CCCGGGATGCTACACACAAC | CAGGCCCATGAAGGTGATGT |
| *IL10* ChIP | TTGCCTGGGAAGTGGGTGCA | AGAAGGCATGCACAGCTCAGC |
| *TNF* ChIP | ACCACGCTCTTCTGCCTGCT | TCCATCCCTCCCTATCAGCGCA |
| *PDL1* ChIP | GCTTCCGCCGATTTCACCGA | TCGGGAAGCTGCGCAGAACT |
| *IDO* ChIP | AGGTTGTGTTTCCGGGCTGCT | ACAGGTGGCCGGAGAAGAACA |
| *SOCS1* ChIP | TCGGCCCTGTTTCCCTCTCT | CCTGGCGGCAGAAAGTGGAA |
| *SOCS3* ChIP | GGTCCCGAATCGAAGTCTCCGT | ACTTCACGGCCGCCAACATCT |
| *B2M* ChIP | CTCTCTCGCTCCGTGACT | GCCGAAAGGGGCAAGTAG |
| *MYOD1* ChIP | GTAGGAGAGGCGGGAGAA | TAGGTCTGCGAGGGTCTC |

ChIP, Chromatin immunoprecipitation.

**TABLE E2.** Clinical features of patients with grass pollen allergy

| Patient no. | Age (y) | Sex | Allergic to | Specific IgE* | Symptoms | Other pollen allergies |
|---|---|---|---|---|---|---|
| 1 | 28 | M | Grasses | 3.220 | RC | *Olea* species, *Cupressus* species |
| 2 | 26 | F | Grasses | 0.355 | RC | *Olea* species, *Cupressus* species |
| 3 | 30 | M | Grasses | 0.603 | RC | |
| 4 | 27 | F | Grasses | 3.500 | RC | *Olea* species, *Platanus* species |
| 5 | 57 | M | Grasses | 0.642 | RC-A | *Olea* species, *Cupressus* species |
| 6 | 27 | M | Grasses | 1.300 | RC-A | *Platanus* species, *Betula* species |

*A*, Asthma; *RC*, rhinoconjunctivitis.
*Determined by ELISA as OD at 492 nm.

# ARTICLE III: Allergoid-mannan conjugates imprint tolerogenic features in human macrophages

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To study whether allergoid-mannan conjugates could reprogram monocyte differentiation into macrophages, THP1, a human monocytic cell line, and human monocytes were used. THP1 monocytes were differentiated into MØ in the presence of phorbol myristate acetate (PMA) and then polarized into different phenotypes with IFN/LPS (M-IFN/LPS pro-inflammatory-like), IL4 (M-IL4 pro-allergic-like), IL10 (M-IL10 regulatory-like) or with PM (M-PM). M-PM displayed a phenotype similar to M-IL10. The influence of the presence of PM in the different MØ polarizations was also studied, observing that PM inhibit M-IFN/LPS polarization and favour the generation of M-IL10 regulatory-like MØ under M-IL4 polarization conditions. Besides, the presence of PM during human monocyte differentiation into MØ with GM-CSF also promotes the generation of IL-10-producing MØ. These results provide information about how macrophages can contribute to the tolerogenic effect of allergoid-mannan conjugates being, together with oral DCs, the main targets of the vaccine.

*Herein, this study is presented with the signed consent of all the co-authors. During the research, I participated in the general experimental design, the performing of the experiments, the analysis and discussion of the data, and also in the organization, writing and revision of the manuscript.*

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LETTER

# Allergoid-mannan conjugates imprint tolerogenic features in human macrophages

To the Editor,

Allergen-specific immunotherapy (AIT) is the only treatment for allergic diseases with potential curative capacity.[1] Successful AIT is associated with the induction of functional allergen-specific Tregs, Bregs, and blocking IgG antibodies. AIT also restores the levels and composition of circulating innate immune cells to those observed in healthy individuals.[1] Polymerized allergoid-mannan conjugates (PM) are novel AIT vaccines being currently assayed in phase II clinical trials. PM target dendritic cells (DCs) and generate functional FOXP3$^+$ Tregs *in vitro* and *in vivo*, which is impaired by alum.[2,3] We recently demonstrated that PM reprogram monocytes from healthy donors and allergic patients into tolerogenic DCs via epigenetic and metabolic reprogramming.[4] However, their capacity to regulate macrophage polarization remains unknown. Macrophages (MØ) exhibit sufficient cell plasticity to be able to polarize into different phenotypes, from pro-inflammatory, pathogen-eliminating, and subsequent tissue-damaging to anti-inflammatory or more regulatory profiles.[5] Together with other innate immune cells, MØ play a key role in tolerance induction during AIT.[6]

We first stimulated MØ obtained from THP1 cells with IFN/LPS (M-IFN/LPS pro-inflammatory-like), IL-4 (M-IL4 pro-allergic-like), IL10 (M-IL10 regulatory-like), or with PM (M-PM) (Figure 1A) without affecting cell viability (Figure 1B) to assess the resulting polarization profiles by quantifying the expression of specific markers. M-IFN/LPS significantly increased the expression of *CCR7*, *CD80*, and *IL6* without significant expression of *CD206* or *IL-10* (Figure 1C). M-IL4 were characterized by a higher expression of *CD206* and a lower expression of *IL10* than M-IL10 (Figure 1C). Interestingly, MØ stimulated with PM showed the highest expression of *IL10* gene and very low expression of *CCR7*, *CD80*, *IL6*, and *CD206* (Figure 1C), thus acquiring a phenotype that resembles M-IL10 regulatory MØ (Figure 1D). We also studied the capacity of PM to modulate the MØ polarization (Figure 1E). PM significantly reduced the expression of *CCR7*, *CD80*, and *IL6* induced during M-IFN/LPS polarization without enhancing *CD206* or *IL10* expression (Figure 1F). PM inhibited *CD206* under M-IL4 and M-IL10 polarization and significantly increased *IL10* both under M-IL4 and M-IL10 polarization (Figure 1F), thus demonstrating that PM inhibit M-IFN/LPS polarization and favor the generation of M-IL10 regulatory-like MØ under M-IL4 polarization conditions (Figure 1G). In allergy, M-IFN/LPS contribute to the initiation of inflammatory responses whereas M-IL4 are pro-allergic and are involved in chronic inflammation. Our data suggest that PM may dampen allergic responses by generating regulatory MØ and by favoring the conversion of M-IFN/LPS and M-IL4 into less inflammatory and more regulatory MØ, respectively. The production of IgG4 following AIT triggers *in vitro* conversion of pro-allergic MØ into immunosuppressive phenotypes.[5]

To confirm these data, we generated human MØ from purified CD14$^+$ monocytes with GM-CSF alone (GM-MØ) or in the presence of PM (Mannan-GM-MØ) without affecting cell viability (Figure 2A). Freshly differentiated Mannan-GM-MØ expressed significantly higher mRNA levels of *CD163* and *CCL2* and the tolerogenic molecules *IL10*, *IDO*, *PDL1*, *SOCS1*, and *SOCS3* than conventional GM-MØ (Figure 2B), indicating the generation of MØ with a suppressive phenotype. Supporting these data, Mannan-GM-MØ showed a higher expression of CD14 compared to conventional GM-MØ upon LPS-activation (Figure 2C). LPS-stimulated Mannan-GM-MØ produced significantly lower levels of the pro-inflammatory cytokines TNF-α and IL-6 and significantly higher levels of anti-inflammatory IL-10 than conventional GM-MØ (Figure 2D). The IL-10/TNF-α and IL-10/IL-6 ratios were also significantly higher in Mannan-GM-MØ (Figure 2E), confirming that PM during monocyte differentiation promote IL-10-producing regulatory MØ. Interestingly, Mannan-GM-MØ from allergic patients loaded with native grass pollen allergens increased the generation of FOXP3$^+$ Treg cells compared to GM-MØ (Figure 2F) as well as their production of IL-10 (Figure 2G), indicating that Mannan-GM-MØ are able to induce and expand allergen-specific Treg cells. The presence of PM during MØ differentiation with M-CSF (Mannan-M-MØ) also increases the tolerogenic profile of those regulatory MØ (Figure S1).

Collectively, we uncover a previously unknown capacity of allergoid-mannan conjugates to imprint tolerogenic features in human macrophages, which might well contribute to the tolerogenic responses induced by this novel AIT vaccine. This may be of particular interest when considering oral macrophages, as these cells have been involved in AIT tolerance induction[6] and, together with oral DCs, are the main cell targets of these novel conjugates.[2]

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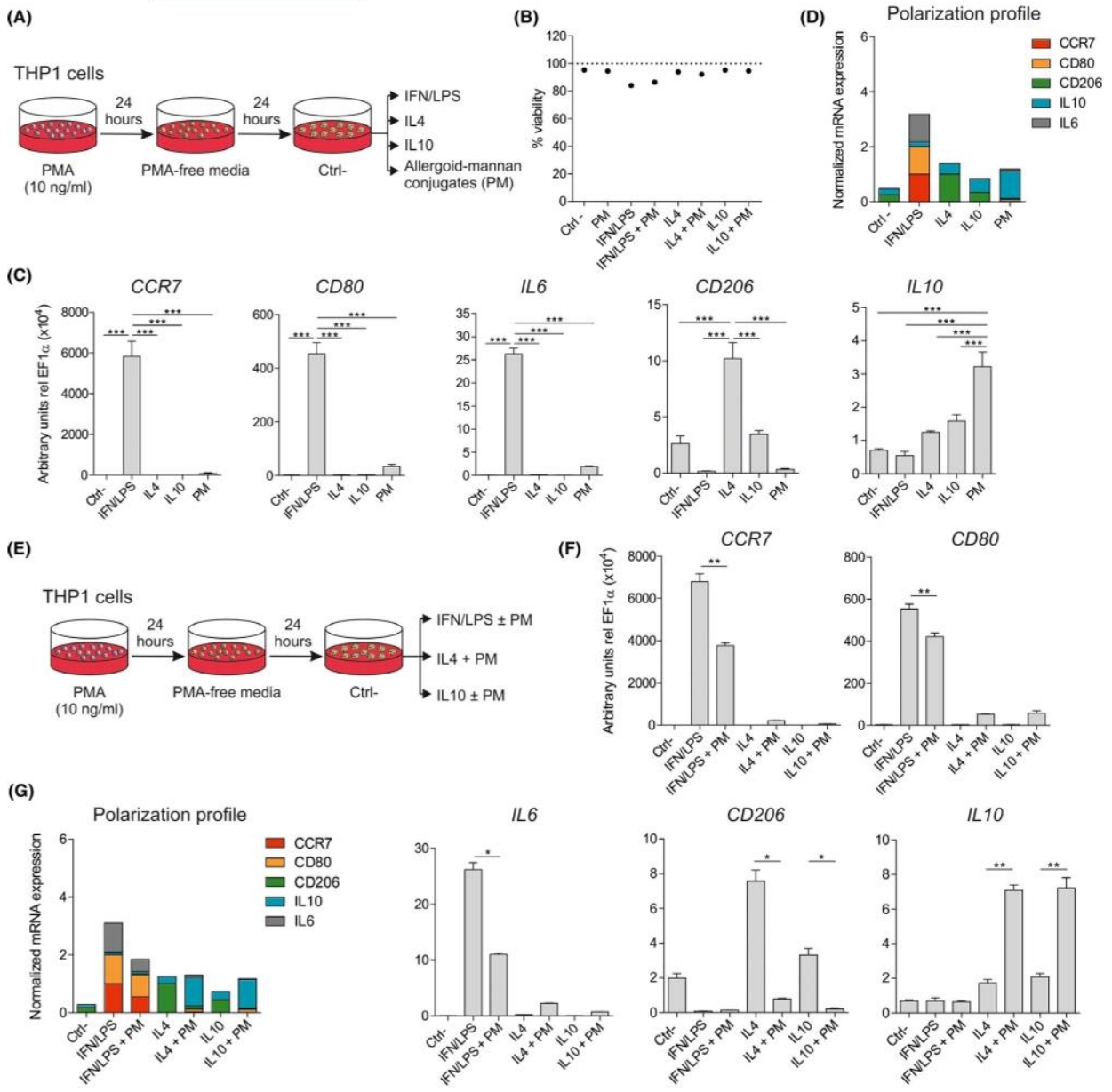


FIGURE 1 (A) THP1 MØ generation and activation protocol in the presence of the different stimuli: medium (Ctrl-), IFN/LPS, IL4, IL10, or PM. (B) Viability. (C) mRNA expression levels of genes in the different MØ phenotypes. (D) Normalized profiles obtained as the mean response patterns normalized to the MØ phenotype with the highest expression of each gene. (E) THP1 MØ generation and activation protocol for M-IFN/LPS, M-IL4, or M-IL10 in the presence or absence of PM. (F) mRNA expression levels of genes in the different MØ phenotypes. (G) Normalized profiles obtained as the mean response patterns normalized to the MØ phenotype with the highest expression of each gene. Results are mean ± SEM of (5–9) independent experiments. One-way ANOVA (ANOVA $p$ value <.05) (C) or Mann-Whitney test (F). *$p < .05$, **$p < .01$, and ***$p < .01$

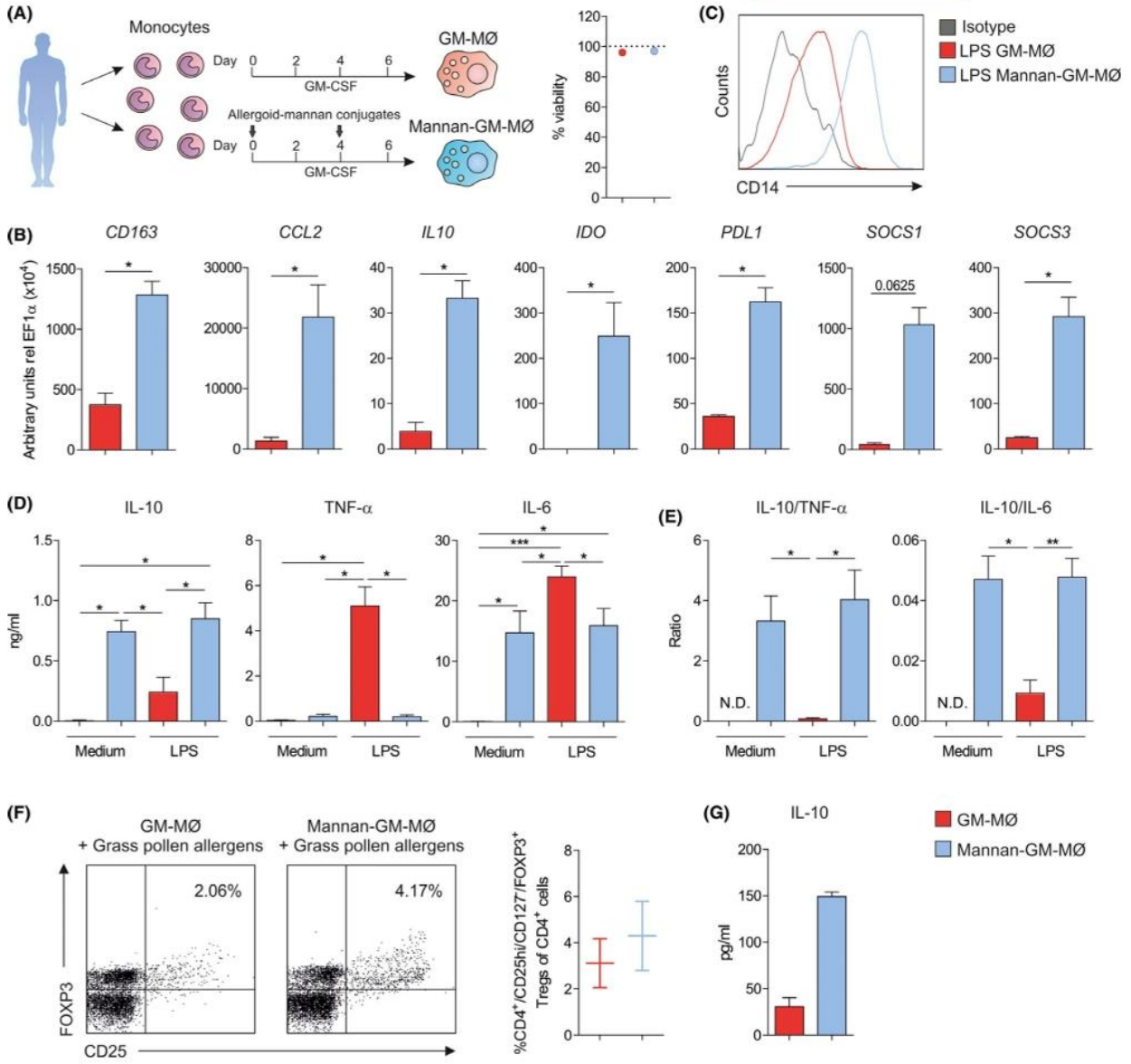


FIGURE 2 (A) Mannan-GM-MØ generation protocol from human monocytes and viability of the generated cells. (B) mRNA expression levels of genes in freshly isolated GM-MØ or Mannan-GM-MØ. (C) Representative histogram of the expression of $CD14^+$ cells after 24 h of LPS-stimulation. (D and E) Cytokine (D) or cytokine ratios (E) produced after 24 h in the presence of medium or LPS. (F and G) Percentage and representative dot plots of induced $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells (gating in lymphocytes) (F) and IL-10 production (G) of peripheral blood lymphocytes after 5 days of coculture with autologous grass pollen-stimulated GM-MØ or Mannan-GM-MØ from allergic patients. Results are mean ± SEM of 2 (A), 6 (B) 4–5 (D and E) and 2 (F and G) independent experiments. Wilcoxon test (B) or one-way ANOVA (ANOVA $p$ value < .05) (D and E). $^*p < .05$, $^{**}p < .01$, and $^{***}p < .01$

**KEYWORDS**

allergen-specific immunotherapy, allergoid-mannan conjugates, human macrophages, immune regulation, innate immunity

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## CONFLICT OF INTEREST

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

## ONLINE REPOSITORY

## MATERIALS AND METHODS

### Material, media and reagents

For cell cultures, we used RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL normocin (InvivoGen, San Diego, Calif), 50 µg/mL penicillin-streptomycin, 1% nonessential amino acids, 1% MEM vitamins and 1 mmol/L sodium pyruvate (Life Technologies, Grand Island, NY). For experiments with THP1 cells, also glucose up to 4.5 g/L was added. LPS (0127:B8) (Sigma-Aldrich, St Louis, Mo) was used. Allergoid-mannan conjugates (Inmunotek) are performed by conjugation of grass pollen *Phleum pratense* allergens with mannan from *Saccharomyces cerevisiae* through the lysine residues from its mannoprotein in a single step. Briefly, glutaraldehyde (25 mmol/L) is added to a solution containing the mixture of allergen and mannan (1:0.5 wt/wt) in PBS for 6 hours at 4ºC in continuous stirring. The reaction is stopped with glycine (1.25 mol/L), followed by tangential flow filtration with distilled water (membrane cut-off, 100 kDa). Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan (PM) were recovered in the concentrated retained fraction (> 100 kDa).

### THP1 macrophages generation and polarization

THP1 cells were grown in culture medium. Prior to the experiments, cells were differentiated to macrophages by incubation with 10 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) in culture medium for 24 hours. They were then washed with medium and grown in culture medium for 24 hours. Then, THP1 macrophages were polarized by incubation overnight with 50 ng/mL IFNγ (Preprotech, Rocky Hill, NJ) plus 100 ng/mL LPS (IFN/LPS), 40 ng/mL IL4 (Preprotech), 50 ng/mL IL10 (Preprotech) or 50 µg/ml allergoid-mannan conjugates (PM). The control population was incubated in culture medium (Ctrl-) without polarizing cytokines. Subsequently, cells were collected, centrifuged and used for phenotype characterization by quantitative real-time RT-PCR analysis. In all the cases, viable cells were counted using Trypan blue with an optical microscope and analyzed by propidium iodide by flow cytometry.

### Primary macrophages generation and polarization

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy donors by Ficoll density gradient centrifugation (800g, 20 minutes). Monocytes were obtained from total PBMC using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To generate human monocyte-derived macrophages, monocytes

were cultured at 0,5x$10^6$cells/mL in 48 well microplates for 6 days with cRPMI medium containing GM-CSF (100 ng/mL, PeproTeck, GM-MØ) or M-CSF (10 ng/mL, PeproTeck, M-MØ). Addition of cytokine took place every 2 days. To generate Mannan-MØ, 50 µg/mL of PM was added at days 0 and 4 of the differentiation. In all the cases, viable cells were counted using Trypan blue with an optical microscope and analyzed by propidium iodide by flow cytometry. Then, macrophages were stimulated with medium (negative control) or LPS (10 ng/ml) for 24 hours, where indicated. Subsequently, cells were collected and centrifuged. Cell-free supernatants were used for cytokine quantification by ELISA and pellets were used for phenotype characterization by flow cytometry or quantitative real-time RT-PCR analysis.

To analyze the induction of allergen-specific Treg cells, unstimulated GM-MØ or Mannan-GM-MØ from allergic patients were cocultured with autologous peripheral blood lymphocytes (PBLs) at a DC/PBL ratio of 1:5 in the presence of native grass pollen allergens (50 µg/mL) to analyze $FOXP3^+$ Treg cells. The 2 grass pollen allergic patients included in this study were previously clinically characterized.[1]

**Cytokine quantification**

Concentrations of IL-10, TNF-α, and IL-6 in cell-free supernatants were quantified by sandwich ELISA using specific ELISA cytokine kits for each one (BD Biosciences, San Jose, CA). In all cases, manufacturer's instructions were followed with minor modifications.

**RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR**

RNA was isolated from harvested cells by using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For mRNA analysis, cDNA was generated with a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR was performed on cDNA by using FastStart Universal SYBR Green Master (Rox; Roche). The sequences of the used primer pairs are listed in **Table 1**. Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems, Foster City, Calif). Data were normalized to EF1α 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 EF1α. To obtain the normalized profiles, we normalized the mean arbitrary units from each gene to the macrophage phenotype with the highest expression of that gene.

### Flow cytometry

Cells were washed with PBS/EDTA 2 mM/0.5% BSA and stained for 15 minutes at room temperature in the darkness with human anti-CD14–PerCP (clone M5E2, dilution 1:50). For analysis of FOXP3 expression in human T cells primed with MØs, cells were first subjected to surface staining with anti-human CD4-PerCP (clone OKT4, dilution 1:100), CD127-PE (clone A019D5, dilution 1:200), and CD25-APC (clone BC96, dilution 1:200) antibodies. After fixation and permeabilization, cells were stained with anti-human FOXP3-Alexa Fluor 488 (clone 259D, dilution 1:30), according to manufacturer's recommendations. The corresponding isotype control was included in each staining. Flow cytometry analysis was performed in a FACSCalibur cytometer (Beckton Dickinson) in the Cytometry and Fluorescence Microscopy Unit at Complutense University of Madrid.

### Statistical analysis

In all experiments, data represent the mean ± SEM of the indicated parameters. Statistical analysis was calculated using one-way ANOVA, whose P value summary was statistically significantly ($P < 0.05$) in all the cases, or Wilcoxon test/ Mann-Whitney test when comparing just two different conditions using Prism software 6.0 (GraphPad Software). Significance is indicated in each figure.

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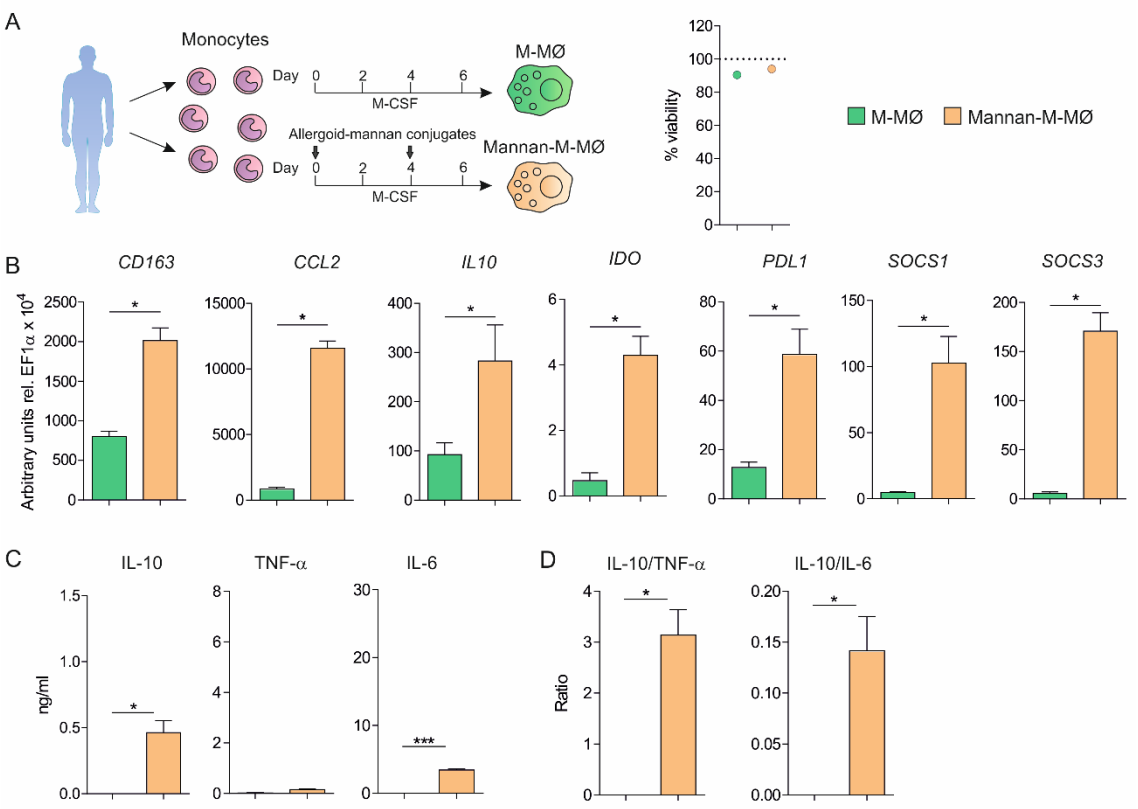


**Figure S1. A**, Mannan-M-MØs generation protocol from human monocytes and viability of the generated cells. **B**, mRNA expression levels of genes in freshly isolated M-MØ or Mannan-M-MØ. **C and D**, Cytokine (C) or cytokine ratios (D) produced after 24 hours in the presence of medium of conventional M-MØ or Mannan-M-MØ. Results are mean ± s.e.m. of 2 (A), 4-6 (B, C and D) independent experiments. Wilcoxon test. **P* < 0.05, and ****P* < 0.01.

**Table 1**. Primer sequences

| Name | Forward | Reverse |
|---|---|---|
| ***CCR7*** | CATGGACCTGGGGAAACCAA | AAAGTTCCGCACGTCCTTCT |
| ***CD80*** | GATAACCTGCTCCCATCCTGG | CTTGGGGCAAAGCAGTAGGT |
| ***CD206*** | ACACAAACTGGGGGAAAGGTT | TCAAGGAAGGGTCGGATCG |
| ***IL10*** | GTGATGCCCCAAGCTGAGA | CACGGCCTTGCTCTTGTTTT |
| ***CD163*** | AAATTACCTGCTCAGCCCACA | ACGTGTCACCATGCTTCACT |
| ***CCL2*** | TCTGTGCCTGCTGCTCATAG | GGGCATTGATTGCATCTGGC |
| ***IDO*** | AGAAGTGGGCTTTGCTCTGC | TGGCAAGACCTTACGGACATCTC |
| ***PDL1*** | AAGATGAGGATATTTGCTGTCTTTATATTC | GTCCTTGGGAACCGTGACAGT |
| ***SOCS1*** | CCCTGGTTGTTGTAGCAGCTT | CAACCCCTGGTTTGTGCAA |
| ***SOCS3*** | CCTCAGCATCTCTGTCGGAAGA | GCATCGTACTGGTCCAGGAACT |
| ***IL6*** | GGTACATCCTCGACGGCATCT | GTGCCTCTTTGCTGCTTTCAC |
| ***EF1α*** | CTGAACCATCCAGGCCAAAT | GCCGTGTGGCAATCCAAT |

# BLOCK B: Study of the capacity of anti-IgE biologicals to immunomodulate the ability of DCs to promote the generation of Treg cells

# ARTICLE IV: Omalizumab restores the ability of human plasmacytoid dendritic cells to induce Foxp3$^{+}$Tregs

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Omalizumab is an anti-IgE mAb approved for the treatment of severe allergic asthma that has been shown to increase the frequency of Treg cells in asthmatic children, which correlates with asthma control. To study the molecular mechanisms by which IgE-mediated signalling in atopic patients could impair the capacity of DCs to generate Treg cells and how this might be influenced by omalizumab, pDCs from atopic donors were used. IgE-FcεRI crosslinking on human pDCs from atopic donors impaired their capacity to polarize Treg cells *in vitro* and decreased IFN-α production and *IDO* expression in TLR9-L-activated pDCs. Omalizumab was able to remove the IgE bound to the FcεRI in the surface of pDCs and restore the effects induced by IgE-FcεRI crosslinking. These results provide novel insights into potential mechanisms by which omalizumab is able to increase Treg cells in asthmatic children.

*This study has been performed with the collaboration of Dr. Jacobo López-Abente and is here presented with his signed consent and that of the rest of co-authors. During the research, I participated in the performing of the experiments, the analysis and discussion of the data, and also in the revision of the manuscript.*

AGORA
RESEARCH LETTER

# Omalizumab restores the ability of human plasmacytoid dendritic cells to induce Foxp3$^{+}$Tregs

*To the Editor*:

Allergic sensitisation and viral respiratory tract infections represent the main risk factors for asthma development and severity. IgE plays a key role in the pathophysiology of allergic asthma and allergic multimorbidities [1, 2]. Omalizumab, a recombinant humanised monoclonal antibody against IgE, has been used to treat allergic asthma in children and adults for many years [3–5]. Omalizumab restores the capacity of human plasmacytoid dendritic cells (pDCs) to produce interferon (IFN)-α, increasing their antiviral activity and reducing viral-induced asthma exacerbations [6, 7]. pDCs prime T-helper cell (Th)1 or Th2 responses depending on the encountered antigens. In both cases, they are able to generate functional regulatory T-cells (Tregs), suggesting that pDCs display intrinsic tolerogenicity [8, 9]. pDCs and Tregs numbers are increased and decreased, respectively, during asthma exacerbations and correlate with the severity of type 2 inflammation [10, 11]. Omalizumab treatment increases the frequency of Tregs in asthmatic children, which correlates with asthma control [12]. The aim of this work is to study the molecular mechanisms by which IgE-mediated signalling in human pDCs from atopic donors could impair their capacity to generate Tregs and how Omalizumab could restore this ability.

We purified pDCs to homogeneity (purity higher than 90% in all the cases without basophil contamination) from peripheral blood of adult atopic donors by magnetic cell sorting (pDC isolation kit II, Miltenyi Biotec) in autoMACS Pro (Miltenyi Biotec). Purified pDCs expressed the high-affinity (FcεR1) but not the low-affinity (CD23) IgE receptor (data not shown). *In vitro* treatment of purified pDCs with Omalizumab (Xolair, Novartis-Pharma-AG) but not with an unrelated human IgG (Privigen, CSL-Behring) significantly reduced the levels of IgE bound to FcεR1 in a specific, dose–dependent manner (figure 1a), without affecting cell viability. This allowed us to mimic *in vitro* the decrease in FcεR1-bound IgE on pDCs demonstrated in Omalizumab-treated patients [13]. Next, we assessed whether IgE-FcεR1 cross-linking (IgE-CL) with an anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA) on human pDCs could influence their ability to prime allogeneic naïve CD4$^{+}$ T-cells (purified from adult blood samples using the Naïve CD4$^{+}$ T-cell isolation kit, Miltenyi Biotec) into Foxp3$^{+}$ Tregs and how Omalizumab could impact this capacity. Purified pDCs stimulated with the Toll-like receptor 9-ligand type B CpG ODN2006 (TLR9-L, Invitrogen) induced higher numbers of CD4$^{+}$CD127$^{low}$CD25$^{+}$Foxp3$^{+}$ Tregs than unstimulated pDCs, which was impaired by IgE-CL in TLR9-L-activated pDCs (figure 1b). Pretreatment of pDCs with Omalizumab restored the capacity of TLR9-L-activated pDCs under IgE-CL to generate CD4$^{+}$CD127$^{low}$CD25$^{+}$Foxp3$^{+}$ Tregs (figure 1b). Cell viability was not affected in any of the assayed pDCs or coculture conditions (data not shown). Supporting these data, the T-cells generated by TLR9-L-activated pDCs under IgE-CL conditions produced lower IL-10 and IL-2 levels than those T-cells generated by TLR9-L-activated pDCs without IgE-CL. The levels of IL-10 and IL-2 produced by the generated T-cells were restored by pretreating pDCs with Omalizumab (figure 1c). Remarkably, the IFN-γ/IL-5 ratio associated with T-cell secretion was lower when pDCs were activated with TLR9-L in the presence of IgE-CL, which was reversed by Omalizumab (figure 1c). Our data show that IgE-CL in TLR9-L-activated pDCs not only impairs the generation of Tregs but also favours Th2 allergic profiles, which is restored by pretreatment of pDCs with Omalizumab. The high dose

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**IgE-FcεR1 cross-linking on human pDCs impairs their capacity to generate regulatory T-cells and *in vitro* omalizumab restores this ability. These findings may pave the way for novel biomarkers to assess omalizumab clinical efficacy and responder patients.** https://bit.ly/2ZQbJ9t

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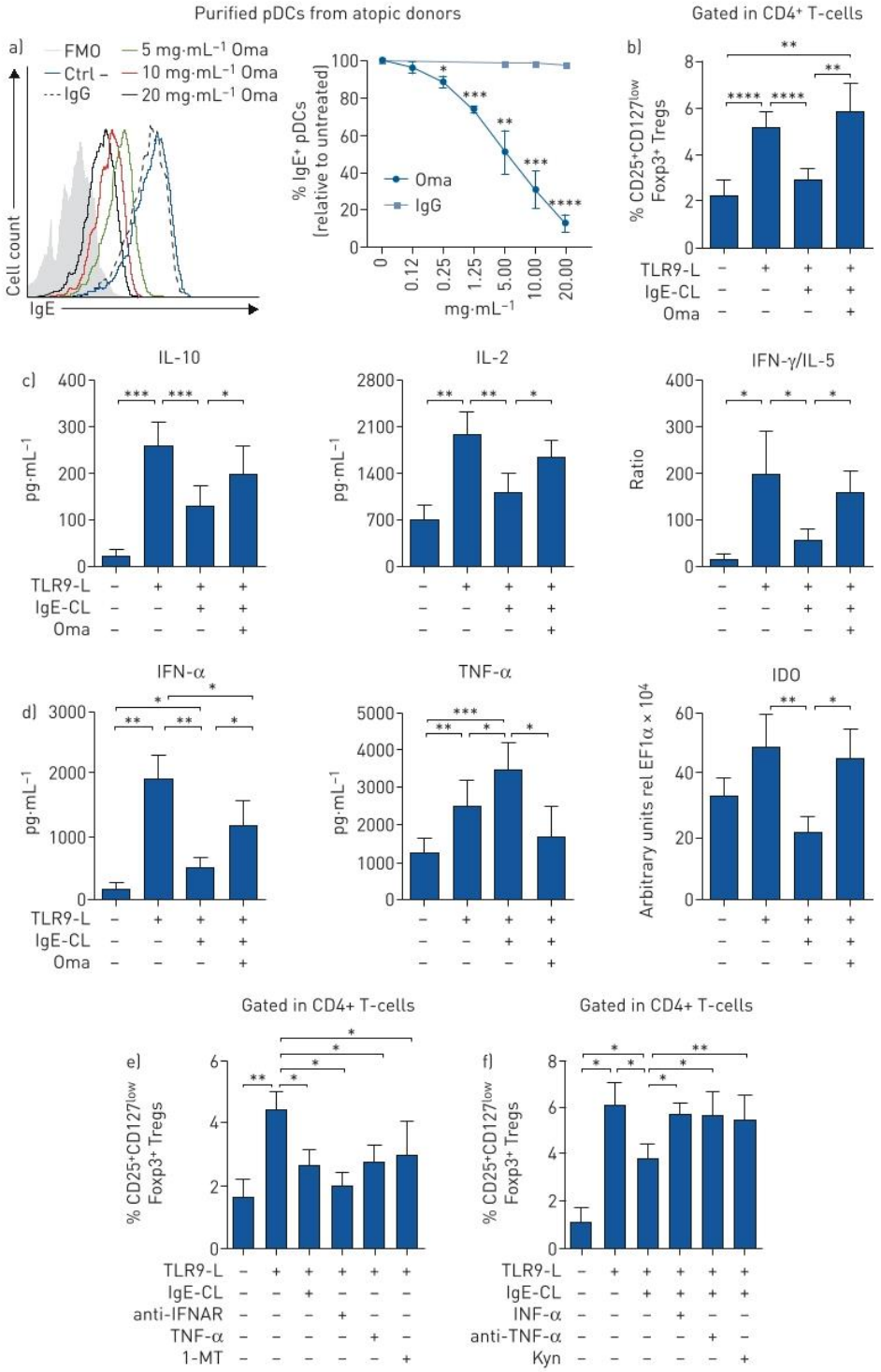


FIGURE 1 Omalizumab removes *in vitro* membrane-bound IgE from purified human plasmacytoid dendritic cells (pDCs) and restores their capacity to induce regulatory T-cells (Tregs). Purified blood pDCs from atopic donors were cultured with different concentrations of Omalizumab (Oma) or intravenous IgG for 18 h. a) Representative histogram and graph showing the levels of IgE bound to pDCs and the frequency of IgE$^+$ pDCs, respectively (n=10). FMO: fluorescence minus one. Omalizumab-treated or untreated purified pDCs

were stimulated with 10 μg·mL$^{-1}$ rabbit anti-human IgE (IgE-FcεR1-crosslinker, IgE-CL) or isotype control in the presence of 2 μM CpG class B TLR9-ligand ODN 2006 (TLR9-L). After 18 h, the pDCs were washed and cocultured with purified allogeneic naïve CD4$^+$ T-cells (1:5 pDC:T-cell ratio, as previously described [9]) for 5 days. b) Graph showing the percentage of induced CD25$^+$CD127$^{low}$Foxp3$^+$ Tregs gated over CD4$^+$ T-cells under the different assayed conditions (n=20) and c) concentration of interleukin (IL)-10 and IL-2 and the interferon (IFN)-γ/IL-5 cytokine ratio in the coculture supernatants (n=12). d) After TLR9-L stimulation, pDCs were washed and the concentration of IFN-α (n=6) and tumour necrosis factor (TNF)-α (n=9) in culture supernatants and mRNA expression of indoleamine-2,3 dioxygenase (IDO) under the different assayed conditions (n=9) were measured. Data are presented as arbitrary units relative to elongation factor 1α (EF1α). Then, the pDCs were cocultured (1:5 pDC:T-cell ratio) in the presence of e) 5 μg·mL$^{-1}$ anti-IFN-α/β receptor blocking antibody (anti-IFNAR), 10 ng·mL$^{-1}$ TNF-α and 250 μM 1-Methyl-D-Tryptophan (1-MT) (n=14), or f) 5 ng·mL$^{-1}$ IFN-α, 5 μg·mL$^{-1}$ anti-TNF-α blocking antibody or 0.5 μM L-kynurenine (Kyn) (n=5) for 5 days and the induced Tregs analysed. Values are given as mean±SEM. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001 in a mixed-effects model with pairwise two-stage Benjamini–Yekutieli's *post-hoc* comparisons.

of Omalizumab needed to detach FcεR1-bound IgE in our study is probably due to the extremely high local concentration of FcεR1-IgE complexes at the single cell level. Omalizumab at the range of clinical concentrations did not detach FcεR1-bound IgE. In real life clinical treatment, Omalizumab traps free IgE, thus gradually reducing FcεR1-bound IgE and FcεR1 expression on pDCs [13]. The Omalizumab concentrations used in our *in vitro* experimental setting will not be achieved in treated patients. Herein, our goal is just to mimic *in vitro* the Omalizumab clinical effect to support the relevance of our main novel fundamental finding: IgE-CL on pDCs breaks Treg induction, which could be restored by Omalizumab.

To gain insights into the molecular mechanisms underlying these effects, we analysed changes in expression levels of molecules involved in T-cell polarisation and tolerogenicity on pDCs. TLR9-L-activated pDCs from atopic donors produced higher levels of IFN-α and tumour necrosis factor (TNF)-α than unstimulated pDCs (figure 1d). Similarly, the mRNA levels of indoleamine-2,3 dioxygenase (IDO), an enzyme involved in tryptophan catabolism, were also increased in TLR9-L-activated pDCs. IgE-CL in TLR9-L-activated pDCs impaired the production of IFN-α and IDO expression, whereas it increased the production of TNF-α (figure 1d). Omalizumab partially or completely restored the levels of all these molecules, suggesting they might represent potential candidates involved in the capacity of IgE-CL to impair the generation of Foxp3$^+$ Tregs by TLR9-L-activated pDCs.

The IFN-α/TNF-α axis and IDO expression have been previously associated with the capacity of pDCs to polarise Tregs [8]. To verify whether the downregulation of IFN-α and IDO, or TNF-α upregulation could be involved in the impaired ability of pDCs to generate Tregs after IgE-CL, we performed functional experiments. Blocking the IFN-α receptor (anti-IFN-α/β receptor blocking antibody (anti-IFNAR), Millipore), inhibiting IDO activity with 1-Methyl D-tryptophan (1-MT, Sigma-Aldrich) and exogenous TNF-α (PreproTech) impaired the capacity of TLR9-L-activated pDCs to induce Tregs (figure 1e). Supporting these data, the capacity of TLR9-L-activated pDCs under IgE-CL to generate Tregs was significantly restored after adding exogenous IFN-α (Biolegend) or kynurenine (a metabolite of downstream tryptophan catabolism that might bypass IDO downregulation; Sigma-Aldrich), or after blocking TNF-α with an anti-TNF-α antibody (Biolegend) (figure 1f). Collectively, our data show that IgE-CL in TLR9-L-activated pDCs reduces the production of IFN-α and IDO expression whereas it increases TNF-α production leading to the impairment of pDCs' capacity to polarise Tregs, which is completely restored by Omalizumab.

In conclusion, we show for the first time that IgE-FcεR1 cross-linking on human pDCs from atopic donors is associated with an impaired capacity of pDCs to polarise Tregs *in vitro*. We provide a molecular mechanism that might well help to explain how Omalizumab treatment increases Tregs frequency in asthmatic children [12]. The induction and maintenance of functional Tregs is essential for healthy immune responses to allergens [8]; therefore, our findings might have important clinical implications also for other allergic conditions [1]. The molecular mechanism described herein might also pave the way for the identification of potential novel biomarkers to assess Omalizumab clinical efficacy and to identify responder patients. Thus, future prospective clinical studies evaluating the capacity of pDCs to induce Tregs and its association with asthma control in Omalizumab treated patients are warranted. In the long run, these studies might well also help to elucidate and monitor whether Omalizumab could display potential long-term disease-modifying capacity for some specific patients.

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# ANNEX I: Study of the capacity of omalizumab, an anti-IgE mAb, to promote the induction of functional regulatory T cells in comparison to corticosteroids.

In order to assess the capacity of omalizumab to immunomodulate the ability of DCs to promote the generation of Treg cells in comparison to corticosteroids, we used pDCs and hmoDCs *in vitro* cultures. These two subsets of DCs are known to induce the generation of Treg cells under the stimulation with TLR9-ligand (TLR9-L, in the case of pDCs) or LPS (in the case of hmoDCs).[134, 189]

## 1. Plasmacytoid DCs as a suitable *in vitro* model

### *1.1. pDCs purification and characterization*

As previously described, pDCs are capable of inducing Treg cells upon TLR9-L (CpG ODN) stimulation, a common stimulus encountered after bacterial infections. Human pDCs were purified to homogeneity from PBMCs from buffy coats by using the *Plasmacytoid Dendritic Cell Isolation Kit II* from Miltenyi Biotec.

The purity of isolated pDCs ranged between 85-95% (**Figure R.18A**). As shown in **Figure R.18B**, purified human pDCs expressed high levels of the high affinity IgE receptor (FcεRI) with minimal expression of the low affinity receptor (CD23).

The levels of plasma IgE from the different donors was quantified by ELISA. There was a positive correlation between plasmatic IgE levels and the frequency of pDCs displaying IgE bound to FcεRI (**Figure R.18C**). The mean value of the percentage of pDCs displaying IgE bound to FcεRI when considering all the assayed donors was 45.71 ± 6.23 (mean ± SEM). Considering this value as a cut-off to differentiate nonatopic vs atopic donors, we selected for further experiments those donors whose pDCs displayed FcεRI-bound IgE frequencies higher than 45% ($IgE^+$ pDCs or atopic donors). A representative dot plot showing the profile of the pDCs from the selected atopic donors in terms of FcεRI-bound IgE is displayed in **Figure R.18D**.

The levels of plasma IgE in the atopic donors included in the study for further detailed analysis were significantly higher than those from nonatopic donors ($IgE^-$ pDCs) (**Figure R.18E**).

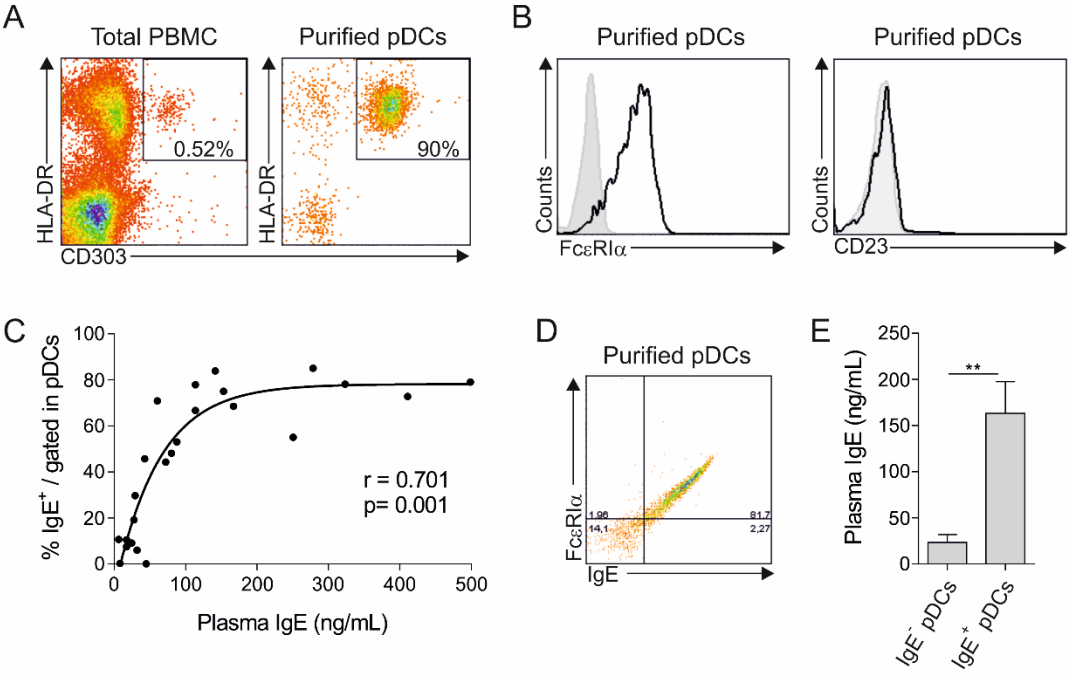


**Figure R.18. Characterization of purified pDCs from human PBMC. A,** Representative dot plots showing the purity of pDCs before and after their isolation. Purified pDCs were HLA-DR$^+$CD303$^+$CD304$^+$CD123$^+$. **B,** Representative histograms of FcεRIα and CD23 expression in purified pDCs. The isotype control is displayed in grey. **C,** Non-linear correlation between the frequency of pDCs with FcεRI-bound IgE (% IgE$^+$ pDCs) and plasma IgE, "r" Spearman correlation coefficient (n = 24). **D,** Representative example of FcεRIα-bound IgE gated on CD303$^+$ pDCs from an atopic donor selected for further experiments. **E,** Plasma IgE levels contained in IgE$^-$ pDCs and IgE$^+$ pDCs donors (n = 24). Those donors whose pDCs were > 45% positive for FcεRI-bound IgE were considered IgE$^+$ pDCs donors. Values are given as mean ± SEM. ***P*<0.01 in Paired Student *t* test.

### *1.2. IgE-mediated FcεRI-crosslinking reduces the expression of functional markers and cytokines essential for Treg cells induction*

IgE-mediated crosslinking initiates downstream signalling in DCs with functional implications.[190] To induce the IgE-FcεRI complex activation, we used a rabbit anti-human IgE (IgE-FcεRI-crosslinker, CL). Purified pDCs were stimulated with 10 µg/mL of CL or the corresponding isotype control (IgG) for 1 hour followed by the addition of 2 µM of CpG type B, ODN2006 (TLR9-L). After 18 hours of stimulation, pDCs gene expression and activation/functional markers expression were analysed.

The stimulation of pDCs with TLR9-L significantly increased the expression of CD-83, CD-86 and PD-L1 compared to unstimulated pDCs (**Figure R.19**). Interestingly, IgE-mediated FcεRI-crosslinking significantly reduced the frequency of CD-83, CD-86, PD-L1 and the expression of HLA-DR in TLR9-L-activated pDCs compared to the isotype control (**Figure R.19**). OX40-L, which is a marker involved in Th2 polarization, displayed an increased expression after TLR9-L stimulation compared to unstimulated pDCs.

Remarkably, after IgE-FcεRI crosslinking in TLR9-L-activated pDCs, the expression of OX40-L is significantly higher compared to unstimulated pDCs (**Figure R.19**).

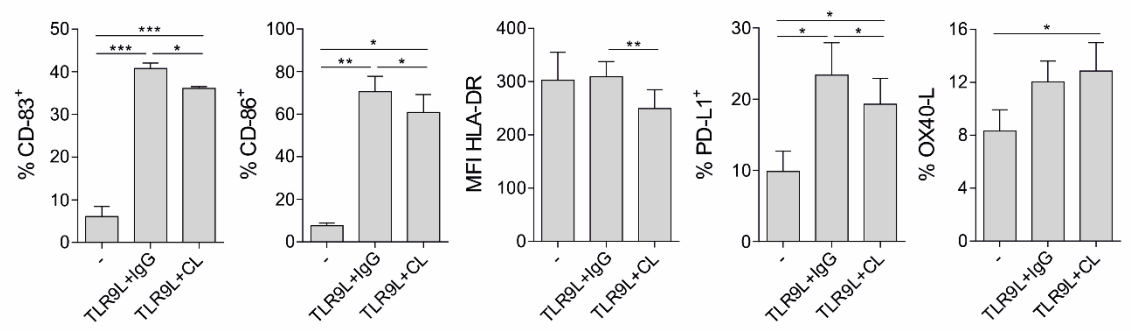


**Figure R.19. IgE-mediated FcεRI-crosslinking alters the expression of markers in IgE⁺ pDCs.** Frequency of CD-83 (n = 4), CD-86 (n = 4), HLA-DR mean fluorescence intensity (MFI, n = 4), frequency of PD-L1 (n = 8) and OX40-L (n = 8) gated in pDCs. Values are given as the mean ± SEM. ***$P$<0.001, **$P$<0.01, *$P$<0.05 in Paired Student $t$ test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

Additionally, TLR9-L-stimulation of pDCs significantly increased the expression of *PDL1* gene compared to unstimulated pDCs. IgE-mediated FcεRI-crosslinking significantly decreased the expression of *ICOSL* and *PDL1* genes in TLR9-L-activated pDCs (**Figure R.20**).

All these molecules, together with *IDO* gene and IFN-α, whose expression and secretion was also decreased after IgE-mediated FcεRI-crosslinking in TLR9-L-activated pDCs as previously described,[191] are known to play a critical role in the generation of Treg cells.[25]

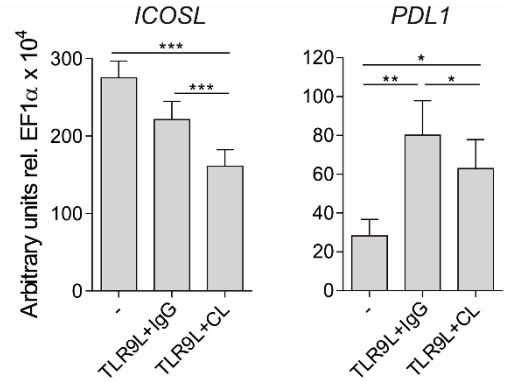


**Figure R.20. IgE-mediated FcεRI-crosslinking alters the expression of genes in IgE⁺ pDCs.** Expression of *ICOSL* and *PDL1* genes (n = 8). Arbitrary units are $2^{-(\Delta Ct)}$ values multiplied by $10^4$, with ΔCt defined as the difference between the cycle threshold value for each gene and elongation factor 1 α (EF1α) as housekeeping gene. Values are given as the mean ± SEM. ***$P$<0.001, **$P$<0.01, *$P$<0.05 in Paired Student $t$ test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

### *1.3. Capacity of omalizumab to promote the generation of Treg cells in comparison to corticosteroids*

We have reported that IgE-mediated FcεRI-crosslinking impaired the capacity of TLR9-L-activated pDCs to induce Treg cells polarization, which was accompanied with a significant decrease of IL-10, IL-2 and IFN-γ production.[191] Moreover, omalizumab

reduced the IgE bound to FcεRI on pDCs, showing a decrease in the IgE MFI in a dose-dependent manner, without affecting cell viability, and displayed the capacity to restore the cytokine signature in TLR9-L-activated pDCs in the presence of IgE-mediated FcεRI-crosslinking.[191]

Inhaled or oral corticosteroids represent the first-line therapy in the effective treatment of persistent allergic asthma, reducing the morbidity and the mortality from this disease. However, long-term corticosteroid therapy (both orally in high doses and systemically administered) has the potential to induce systemic side effects and it is not effective in all the patients.[192] The use of omalizumab in patients with severe allergic asthma reduces the corticosteroid burden and improves the asthma control.[193, 194] Dexamethasone is one of the corticosteroids used for asthma treatment.

To check whether omalizumab could restore the capacity of pDCs to generate Treg cells in comparison with the effects exerted by corticosteroids, pDCs were treated with 10 mg/mL of omalizumab for 24 hours. pDCs were then treated with 1 µM of dexamethasone, CL and TLR9-L stimulation. After 24 hours, pDCs were washed and cocultured with allogeneic naïve $CD4^+$ T cells for 5 days. As expected, there was a significant increase of Treg cells associated to the TLR9-L stimulation (**Figure R.21**). As previously described, IgE-FcεRI crosslinking significantly decreased the frequency of induced Treg cells, which was reverted by omalizumab. However, dexamethasone treatment abolished the upregulation of the induction of Treg cells by TLR9-L stimulation in pDCs (**Figure R.21**). Summing up, omalizumab is capable of restoring pDCs capacity to generate Treg cells under IgE-FcεRI-crosslinker stimulation, whereas dexamethasone suppresses pDCs function.

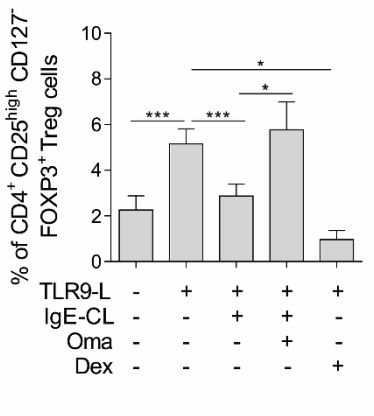


**Figure R.21. Omalizumab restores the capacity of pDCs to induce Treg cells.** $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells generated after 5 days by pDCs from atopic donors treated with the indicated conditions (gating in lymphocytes, n = 7). Values are given as the mean ± SEM. ***$P$<0.001, *$P$<0.05 in Paired Student $t$ test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker, *Oma* Omalizumab, *Dex* Dexamethasone.

## 2. Human monocyte-derived dendritic cells as a suitable *in vitro* model

### *2.1. Study of the expression of FcεRI and CD23 on monocytes and in the generated hmoDCs*

DCs are equipped with FcεRI and CD23. Therefore, we firstly evaluated the expression of both receptors in human monocytes and hmoDCs by flow cytometry and qPCR. Freshly isolated monocytes purified from PBMCs did not express CD23 and only a small fraction expressed FcεRIα (**Figure R.22A**). All the FcεRIα$^{+}$ monocytes displayed membrane-bound IgE (**Figure R.22A**). Once the monocytes were differentiated into hmoDCs in the presence of IL-4 and GM-CSF, around 70% hmoDCs expressed CD23 and around 5% expressed FcεRIα. Although the mean fluorescence intensity of FcεRIα was lower in hmoDCs compared to monocytes, the percentage of FcεRIα$^{+}$ cells was higher in hmoDCs (around 5%) than in monocytes (around 2%) which was also corroborated by qPCR (**Figure R.22B**). Importantly, hmoDCs did not show endogenous membrane-bound IgE. This is relevant since it will allow us to control the addition of exogenous IgE to assess IgE-mediated FcεRIα-crosslinking signalling.

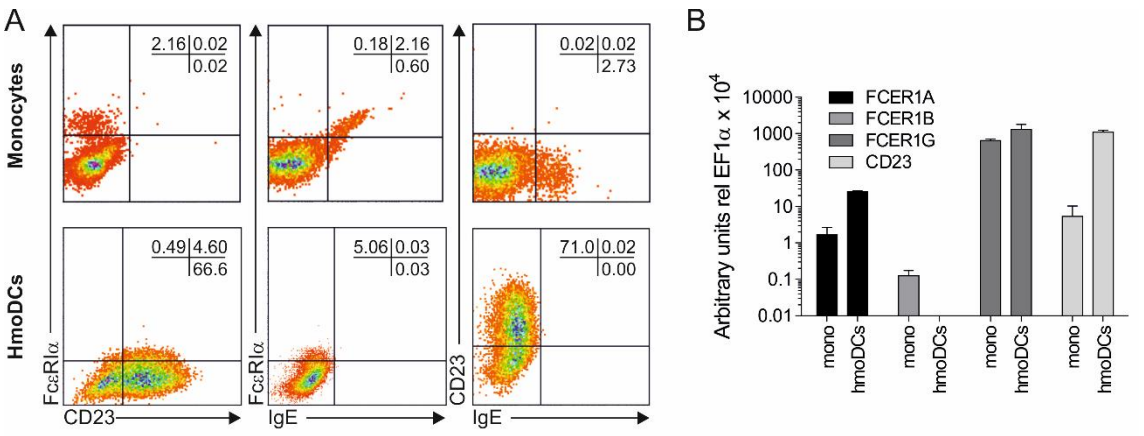


**Figure R.22. FcεRIα and CD23 expression in human monocytes and hmoDCs.** Monocytes were purified from human PBMCs using anti-CD14 magnetic beads. High monocyte purity was achieved (>90%, as determined by the expression of CD14). Monocytes were cultured in complete RPMI medium supplemented with IL-4 and GM-CSF for 6 days to obtain hmoDCs. **A,** FcεRIα$^{+}$, CD23$^{+}$, and IgE$^{+}$ monocytes and hmoDCs analysed by flow cytometry. **B,** *FCER1A*, *FCER1B*, *FCR1G* and *CD23* gene expression in monocyte and hmoDCs analysed by qPCR (n = 2). Arbitrary units are $2^{-(\Delta Ct)}$ values multiplied by $10^4$, with ΔCt defined as the difference between the cycle threshold value for each gene and EF1α as housekeeping gene. Values are given as the mean ± SEM.

In contrast to mast cells and basophils, we verified that monocytes and hmoDCs lack the FcεRIβ chain expression by qPCR (**Figure R.22B**). Both monocytes and hmoDCs express the functional trimeric receptor (α and two γ chains) as it has been already reported in human DCs.

Collectively, these results show that *in vitro* differentiation of human monocytes into DCs generates a population of hmoDCs expressing both FcεRIα and CD23 without membrane-bound IgE.

Then, we analysed the capacity of hmoDCs to bind IgE *in vitro*. For that, human IgE was added to the culture of hmoDCs and FcεRIα$^+$, CD23$^+$ and IgE$^+$ hmoDCs were monitored by flow cytometry at 1, 4, and 18 hours after IgE supplementation. While the frequency of IgE$^+$CD23$^+$ hmoDCs started to decrease immediately after exogenous IgE supplementation, the frequency of IgE$^+$FcεRIα$^+$ hmoDCs increased, reaching maximum values 18 hours after IgE supplementation (**Figure R.23A-B**).

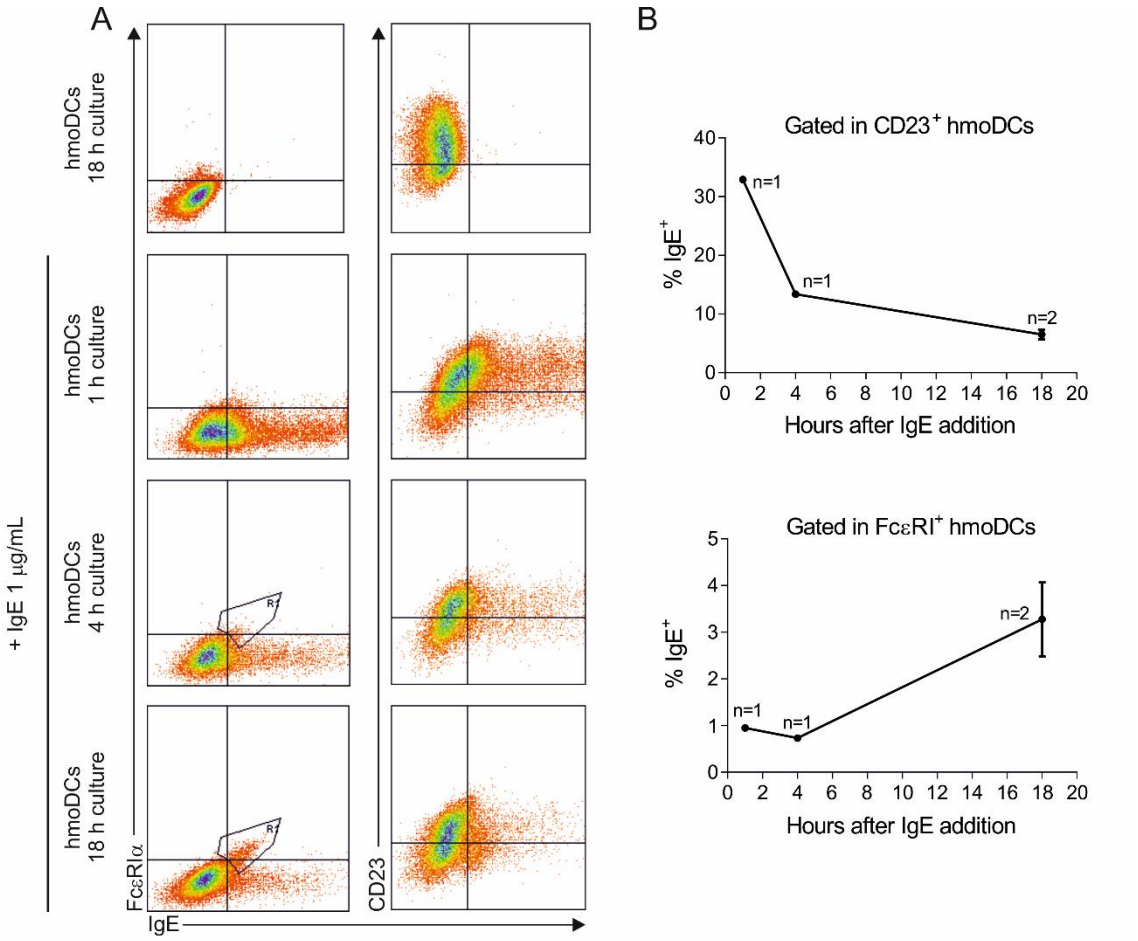


**Figure R.23. Kinetics for IgE-binding in hmoDCs.** HmoDCs were cultured in RPMI medium and supplemented with 1 µg/mL of human IgE. FcεRIα$^+$, CD23$^+$, and IgE$^+$ hmoDCs were analysed by flow cytometry at 1, 4, and 18 hours after IgE supplementation. **A,** Representative dot plots and **B,** graphs showing IgE$^+$CD23$^+$ and IgE$^+$FcεRIα$^+$ hmoDCs at different time points. Values are given as the mean ± SEM.

### *2.2. Effect of IgE-mediated FcεRI-crosslinking on maturation and cytokine production in hmoDCs*

As mentioned above, IgE-mediated FcεRI-crosslinking is required to initiate signalling in human DCs. Therefore, the rabbit anti-human IgE (IgE-FcεRI-crosslinker, CL) was used as in the pDCs model. IgE-FcεRI-crosslinker was added to hmoDCs one hour after IgE supplementation together with LPS as an activation stimulus. After 18 hours, hmoDCs gene expression, activation/functional markers expression and supernatant cytokines were analysed.

Stimulation of IgE-loaded hmoDCs with LPS increased the expression of CD-83, CD-86, HLA-DR and PD-L1 compared to unstimulated hmoDCs. IgE-mediated FcεRI-crosslinking induced an increase in the frequency of CD-83 and a decrease in the percentage of PD-L1$^{+}$ hmoDCs, with no significant differences in the rest of markers analysed compared to LPS-activated hmoDCs (**Figure R.24**).

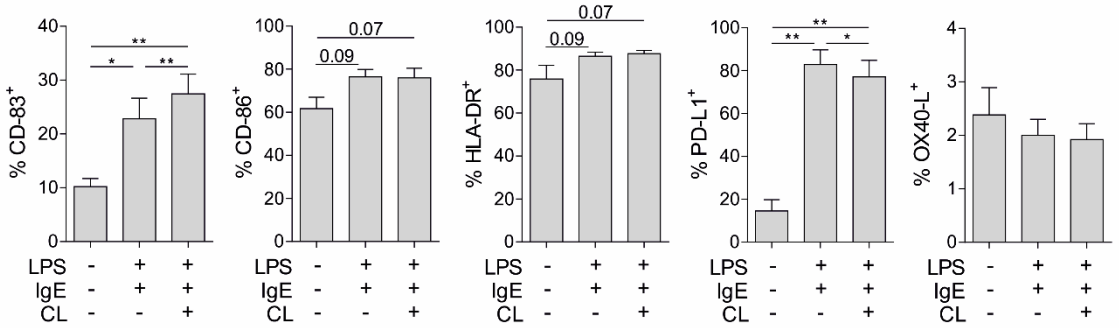


**Figure R.24. IgE-mediated FcεRI-crosslinking alters the expression of markers in hmoDCs.** HmoDCs were supplemented with 1 µg/mL of IgE for 1 hour; then, 10 µg/mL CL and 100 ng/ml of LPS were added to the culture. Graphs showing the percentage of different surface markers determined by flow cytometry after 18 hours of activation (n = 4-8). Values are given as the mean ± SEM. ***P*<0.01, **P*<0.05 in Paired Student *t* test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

The expression of *PDL1* and *IDO* genes was significantly increased after LPS stimulation of hmoDCs compared to unstimulated hmoDCs, while there was no difference in *ICOSL* expression. However, there were no significant differences in the expression of any of the assayed genes when comparing IgE-FcεRI-crosslinker and its isotype control conditions (**Figure R.25**).

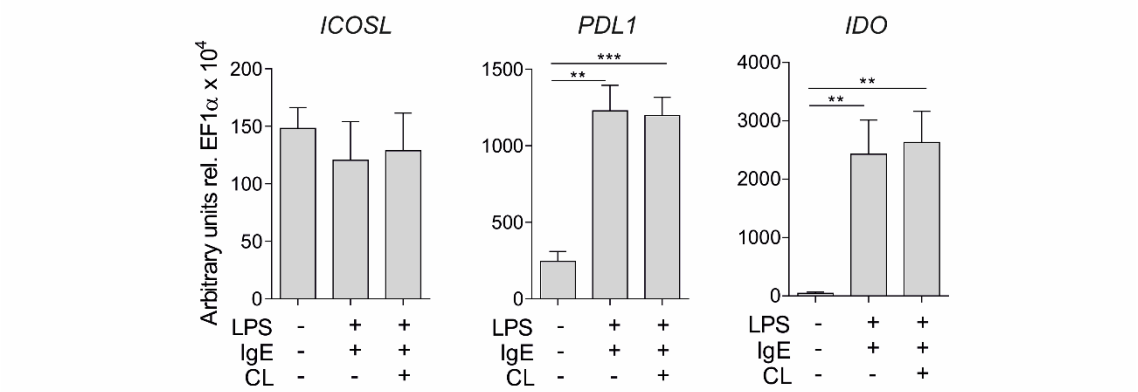


**Figure R.25. IgE-mediated FcεRI-crosslinking does not change gene expression in hmoDCs.** Expression of *ICOSL*, *PDL1* and *IDO* genes of hmoDCs after LPS plus IgE+CL stimulation compared to LPS plus IgE+isotype control (IgG). Arbitrary units are $2^{-(\Delta Ct)}$ values multiplied by $10^4$, with ΔCt defined as the difference between the cycle threshold value for each gene and elongation factor 1 α (EF1α) as housekeeping gene. Values are given as the mean ± SEM. ***$P$<0.001, **$P$<0.01 in Paired Student $t$ test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

Regarding the production of cytokines, LPS-stimulated hmoDCs produced significantly higher levels of TNF-α and IL-10 than unstimulated hmoDCs. After IgE-FcεRI-crosslinking, there was a tendency to higher TNF-α production and no differences on IL-10 (**Figure R.26**) or other assayed cytokines (data not shown).

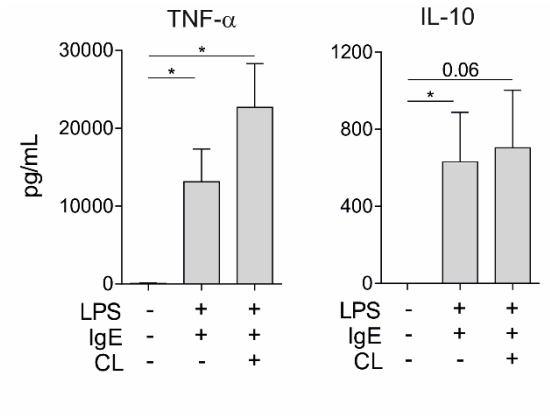


**Figure R.26. IgE-mediated FcεRI-crosslinking induces slight changes in cytokine production by hmoDCs.** TNF-α and IL-10 cytokines measured by ELISA (n = 5). Values are given as the mean ± SEM. *$P$<0.05 in Paired Student $t$ test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

## *2.3. Capacity of hmoDCs stimulated with IgE-FcεRI-crosslinker to generate Treg cells*

The induction and maintenance of functional Treg cells is essential for healthy immune responses to allergens.[25] To analyse the induction of Treg cells by hmoDCs after IgE-FcεRI crosslinking, we incubated hmoDCs with IgE, LPS and CL, washed and cocultured for 5 days with allogeneic naïve $CD4^+$ T cells in a 1:5 ratio (hmoDCs: naïve T cell ratio). The production of cytokines by T cells was analysed in the coculture supernatant. As shown in **Figure R.27**, LPS-stimulated hmoDCs induced more IFN-γ-, IL-5-, and IL-2-producing T cells compared to unstimulated hmoDCs. Besides, there were no statistical

differences in IFN-γ and IL-2 levels between the IgE-FcεRI-crosslinker and isotype control conditions. IL-10 was undetectable in all of the assayed conditions (**Figure R.27**). Interestingly, there was a significant decrease in IL-5 levels produced by T cells cocultured with LPS-activated DCs after IgE-FcεRI-crosslinker, but the values in all the conditions were very low and close to the limit of detection. All the cytokines, including IL-2, had a tendency to decreased levels under IgE-FcεRI-crosslinker conditions, highlighting that there may be decreased T-cell activation, as already shown in pDCs (**Figure R.27**).

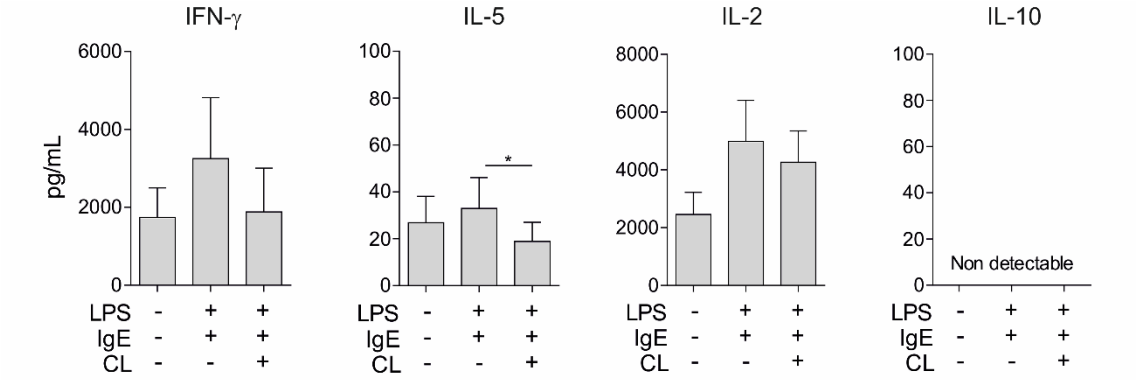


**Figure R.27. IgE-mediated FcεRI-crosslinking in LPS-activated hmoDCs decreases T cells cytokine production.** IFN-γ (n = 7), IL-5 (n = 6), IL-2 (n = 6) and IL-10 (n = 5) in culture supernatant, measured by ELISA. Values are given as the mean ± SEM. **P*<0.05 in Paired Student *t* test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

Although the influence of IgE-mediated FcεRI-crosslinking in LPS-activated hmoDCs is slight in terms of cytokine production and gene expression, we have corroborated that it impaired the capacity of hmoDCs stimulated with LPS to induce Treg cells polarization (**Figure R.28**), similarly to the effect observed with IgE-mediated FcεRI-crosslinking in pDCs.

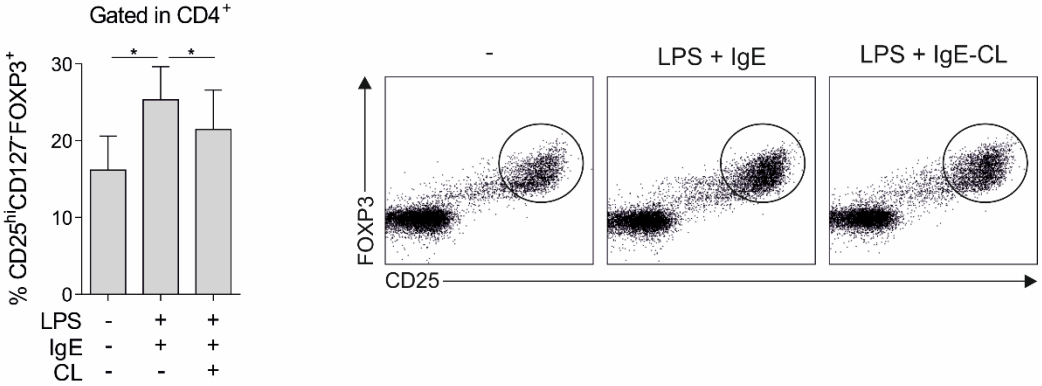


**Figure R.28. IgE-mediated FcεRI-crosslinking impairs LPS-activated hmoDCs capacity to induce Treg cells.** Graph and representative dot plots of $CD127^{-}CD25^{high}FOXP3^{+}$ Treg cells frequency gated over $CD4^{+}$ T cells after coculture (n = 7). Values are given as the mean ± SEM. **P*<0.05 in Paired Student *t* test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker.

### *2.4. Omalizumab blocks IgE and restores hmoDCs capacity to induce Treg cells*

Omalizumab, a recombinant humanized monoclonal antibody against IgE, has been used to treat allergic asthma in children and adults for many years.[15] Omalizumab restores the capacity of human pDCs to produce IFN-α, increasing antiviral activity and reducing viral-induced asthma exacerbations.[25, 195-197]

Our data show that IgE-FcεRI-crosslinker activation on hmoDCs impairs their capacity to generate Treg cells in coculture experiments. Next, we evaluated whether omalizumab was capable of blocking IgE binding to FcεRI in hmoDCs. For that, IgE was incubated alone or together with omalizumab for 1 hour prior to its addition to hmoDCs. Afterwards, the cells were incubated with the stimuli for 1 hour and IgE binding to hmoDCs was analysed by flow cytometry. We observed that omalizumab completely blocked IgE binding to FcεRI in hmoDCs (**Figure R.29A**). Next, we wanted to assess if omalizumab was able to reverse the IgE-mediated FcεRI-crosslinking effect over Treg cells induction. As it is shown in **Figure R.29B**, omalizumab significantly restored hmoDCs capacity to generate Treg cells.

It is known that omalizumab treatment increases the frequency of Treg cells in asthmatic children, which correlates with asthma control.[198] Our experiments can provide a molecular mechanism by which that increase occurs both in hmoDCs and pDCs.

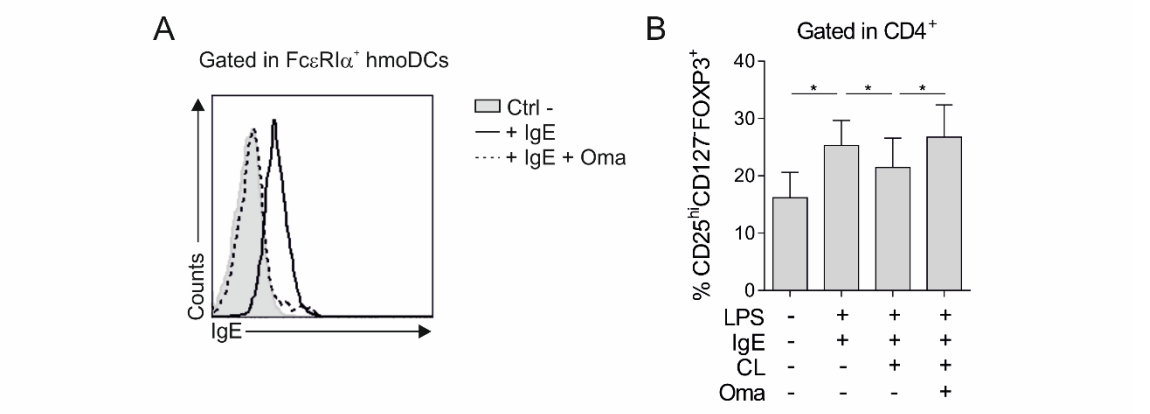


**Figure R.29. Omalizumab blocks IgE binding to FcεRI in hmoDCs, restoring hmoDCs capacity to generate Treg cells.** HmoDCs were cultured with 1 µg/mL of IgE or IgE plus 10 µg/mL of omalizumab for 1 hour. Then, **A,** FcεRIα$^+$, and IgE$^+$ hmoDCs were analysed by flow cytometry. **B,** Cells were washed and stimulated with 100 ng/mL of LPS and 10 µg/mL of the IgE-FcεRI-crosslinker (CL) or isotype control (IgG) overnight. Afterwards, hmoDCs were washed again and cocultured with allogeneic naïve CD4$^+$ T cells in a 1:5 ratio for 5 days. The graph shows CD127$^-$CD25$^{high}$FOXP3$^+$ Treg cells frequency gated over CD4$^+$ T cells after coculture (n = 7). Values are given as the mean ± SEM. *$P$<0.05 in Paired Student $t$ test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker, *Oma* Omalizumab.

## 2.5. Effect of Dexamethasone on maturation and cytokine production in hmoDCs

We also studied the effects of corticosteroids in hmoDCs phenotype and their capacity to induce functional T cell responses. For that, hmoDCs were incubated with 1 µM dexamethasone for 1 hour prior to the addition of LPS. After 18 hours of activation with LPS, hmoDC activation markers and supernatant cytokines were analysed.

The presence of dexamethasone induced a decrease tendency in the frequency of CD-83$^+$ and CD-86$^+$ cells in LPS-activated hmoDCs, whereas the expression of the MHC class II HLA-DR was not affected in any of the assayed conditions (**Figure R.30**).

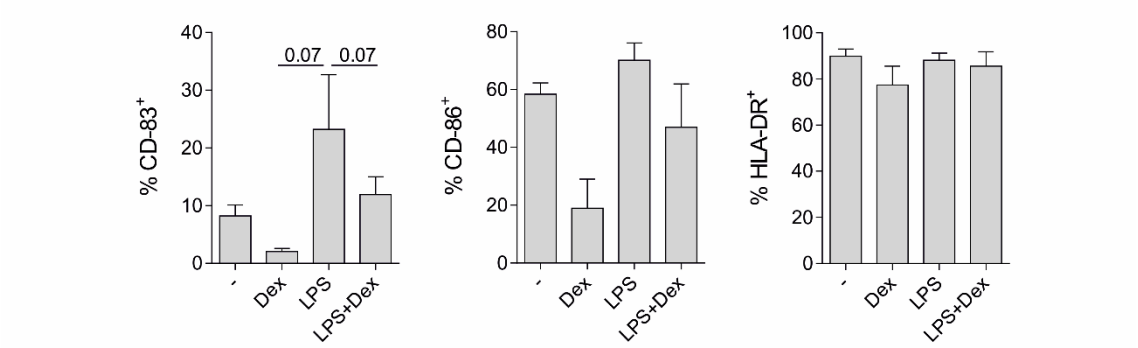


**Figure R.30. Dexamethasone inhibits the activation of LPS-stimulated hmoDCs.** Graphs showing % of CD83$^+$, CD86$^+$, and HLA-DR$^+$ hmoDCs (n = 4). Values are given as the mean ± SEM. Paired Student *t* test comparing multiple conditions.

Then, we wanted to study the influence of dexamethasone in the production of cytokines by LPS-stimulated hmoDCs. We observed that dexamethasone significantly inhibited the secretion of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and the anti-inflammatory cytokine IL-10 (**Figure R.31**). It has been previously described that dexamethasone is able to induce the generation of tol-DCs, which correlates with the results observed in this study.[199]

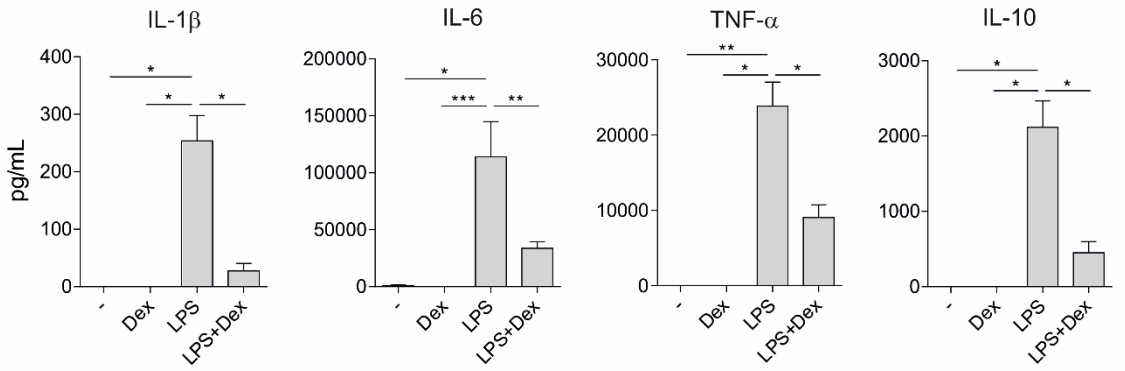


**Figure R.31. Dexamethasone inhibits cytokine secretion of LPS-activated hmoDCs.** IL-1β (n = 3), IL-6 (n = 9), TNF-α (n = 6) and IL-10 (n = 3) cytokines produced by hmoDCs measured by ELISA. Values given the mean ± SEM. ***$P$<0.001, **$P$<0.01, *$P$<0.05 in Paired Student *t* test comparing multiple conditions.

### *2.6. Effect of dexamethasone on hmoDCs capacity to generate Treg cells*

Next, we analysed the capacity of dexamethasone-treated hmoDCs to induce T-cell responses and promote the generation of Treg cells. HmoDCs were incubated with dexamethasone and LPS, washed and cocultured with allogeneic naïve $CD4^+$ T cells as previously described. The production of cytokines by T cells was analysed in the coculture supernatant showing that there was a tendency to reduce the levels of all the assayed cytokines (IL-10, IFN-γ and IL-5) (**Figure R.32A**).

Remarkably, even though there were no statistical differences in the amount of IL-10, there was a tendency to decreased levels when LPS-activated hmoDCs were treated with dexamethasone, highlighting that their capacity to induce Treg cells might be compromised. To analyse that, we determined the percentage of $CD4^+CD127^-CD25^{high}FOXP3^+$ T cells induced in the cocultures and we observed that dexamethasone significantly decreased the capacity of LPS-activated hmoDCs to generate Treg cells (**Figure R.32B**).

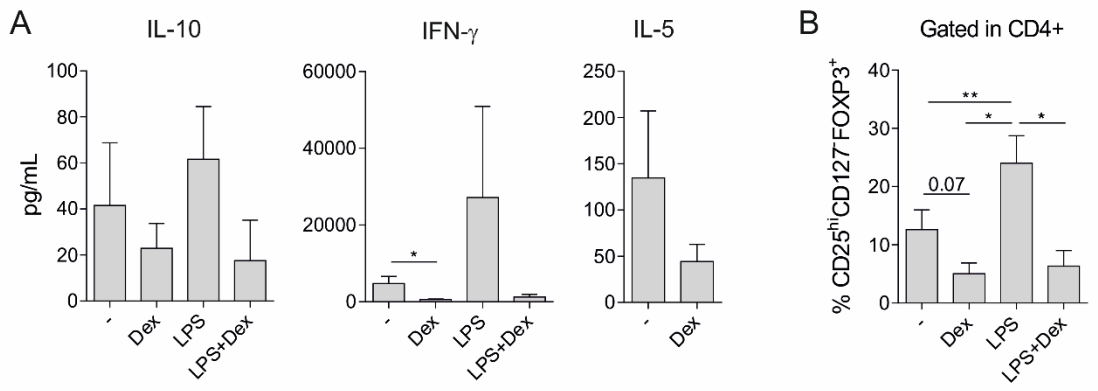


**Figure R.32. Dexamethasone impairs LPS-activated hmoDCs capacity to induce Treg cells. A,** IL-10 (n = 6), IFN-γ (n = 8) and IL-5 (n = 4) cytokines in coculture supernatant, measured by ELISA. **B,** Graph of $CD127^-CD25^{high}FOXP3^+$ Treg cells frequency gated over $CD4^+$ T cells after coculture (n = 11). Values are given as the mean ± SEM. ***P*<0.01, **P*<0.05 in Paired Student *t* test comparing multiple conditions.

### *2.7. Comparison between the effect of omalizumab and dexamethasone over the hmoDCs capacity to generate Treg cells*

One of the aims of this study was to compare the effect of omalizumab and corticosteroids treatment (dexamethasone) over the hmoDCs capacity to generate Treg cells. We have already observed that omalizumab significantly restored hmoDCs capacity to generate Treg cells (**Figure R.29B**). Dexamethasone inhibited the LPS-activated hmoDCs capacity to induce Treg cells, obtaining similar induction levels to the observed in the unstimulated condition (**Figure R.32B**). Therefore, the corticosteroid dexamethasone exhibits strong immunosuppressive properties and inhibits the capacity

of human DCs to induce Treg cells, while omalizumab displays immunomodulatory properties in human DCs and promotes the generation of Treg cells, which are essential to restore healthy immune responses to allergens.

In summary, we provide novel insights into the molecular mechanisms by which IgE-FcεR1 crosslinking on human pDCs impairs their capacity to generate Treg cells and how omalizumab could restore this ability *in vitro*. Our findings might well help to elucidate the potential long-term disease-modifying capacity of omalizumab and to pave the way for the identification of novel potential biomarkers to assess clinical efficacy and responder patients in severe allergic asthma. Thus, prospective follow-up studies evaluating pDCs capacity to induce Treg cells and its association with asthma control in omalizumab treated patients is warranted. These studies will help to elucidate omalizumab potential long-term disease-modifying capacity.

# ANNEX II: Study of the capacity of Ligelizumab compared to Omalizumab to condition the capacity of DCs to polarize T cell responses, with special focus on the generation of Treg cells.

Ligelizumab, a new anti-IgE mAb with much higher affinity than omalizumab ($K_D$ of 139 pM vs 6-8 nM), has already shown dose-dependent and time-dependent suppression of free IgE, basophil FcεRI, and basophil surface IgE.[105] As described above, omalizumab restores the capacity of pDCs to produce high levels of type I IFN-α and to induce the generation of Treg cells. However, the capacity of ligelizumab compared to omalizumab to restore the ability of DCs to generate Treg cells as well as the potential physiological and clinical relevance of such effects in the context of allergic diseases remain fully unknown.

## 1. Plasmacytoid DCs *in vitro* model for the study of ligelizumab

To assess the capacity of ligelizumab to remove the IgE bound to DCs, we used atopic pDCs displaying FcεRI-bound IgE frequencies higher than 45%, as previously described.

pDCs were treated for 18 hours with 0.25, 1.25 and 5 mg/mL of ligelizumab or omalizumab, and the expression of IgE was analysed by flow cytometry. In the representative example, untreated pDCs (Ctrl -) displayed a 50.0% of $IgE^+$ cells. The treatment with IgG at the highest dose tested (5 mg/mL) did not reduce the levels of IgE on the surface of the cells (**Figure R.33**). *In vitro* treatment of pDCs with ligelizumab for 18 hours at increasing doses demonstrated that ligelizumab, unlike omalizumab, was not able to remove the IgE bound to FcεRI in the surface of pDCs at any of the concentrations tested (**Figure R.33**). This result can be explained due to the different epitopes involved in IgE binding. As shown in **Figure I.10**, it has been reported that ligelizumab and omalizumab recognize distinct epitopes of the IgE Cε3 domain, with some overlap. In addition, ligelizumab binding site to IgE overlaps with FcεRI interaction site, while omalizumab binds more distantly to FcεRI.[101] Therefore, ligelizumab binding site on IgE is not available when IgE is bound to FcεRI and the two anti-IgE mAb display different capacity to inhibit the interaction between IgE and FcεRI or CD23 receptors.

The main objective of this study was to compare the effects of ligelizumab and omalizumab on the functional properties of DCs to polarize T cells. As ligelizumab does not remove the IgE bound to the surface of pDCs, the model previously used to analyse the capacity of omalizumab to restore the induction of Treg cells by TLR9-L-activated

pDCs after FcεRI-IgE crosslinking is not appropriate for ligelizumab studies. Consequently, we decided to optimize a new *in vitro* protocol with pDCs in which IgE was removed from the membrane previous to the addition of ligelizumab or omalizumab.

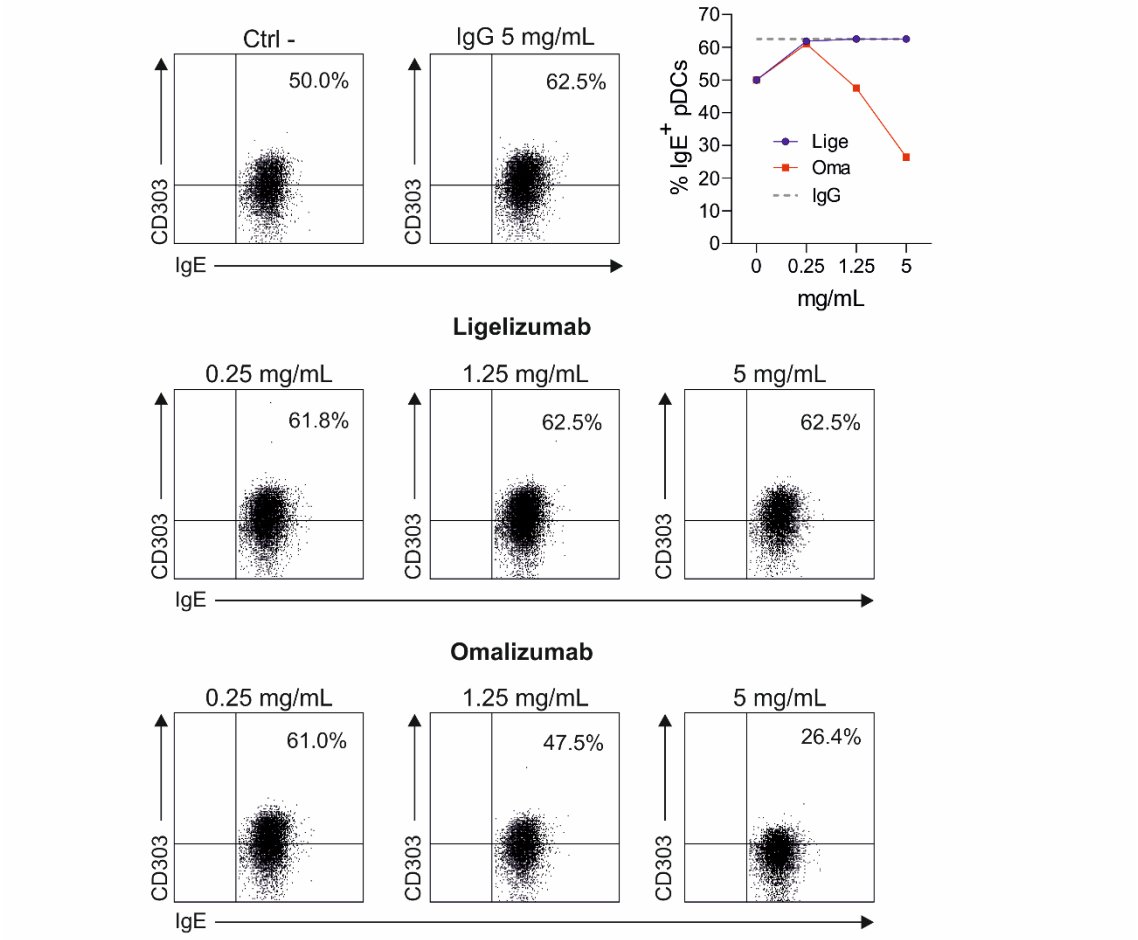


**Figure R.33. Ligelizumab does not detach IgE in pDCs from atopic donors.** pDCs were treated with the indicated concentrations of ligelizumab and omalizumab for 18 hours and the expression of IgE$^+$ cells was analysed by flow cytometry. *Lige* Ligelizumab, *Oma* Omalizumab.

## 2. Stripping of IgE with lactic acid

In order to use pDCs from atopic donors and compare omalizumab and ligelizumab effects, IgE$^+$ pDCs were stripped to remove all the IgE bound to FcεRI on the membrane. It has been demonstrated that treatment of basophils with lactic acid successfully removed a large part of the IgE antibodies originally bound to FcεRI on the surface of the human basophils.[200] Therefore, we initially tried this protocol, which had never been explored in pDCs before.

Purified IgE$^+$ pDCs were incubated with lactic acid for 1 minute, rapidly washed, and the levels of IgE on the surface of the pDCs were quantified by flow cytometry. As shown in **Figure R.34**, after 1 minute of stripping with lactic acid, there was a significant

reduction of the IgE bound to the membrane of the pDCs, but as indicated in this representative example, there was still around 20% of IgE$^+$ pDCs under the assayed conditions. This indicates that IgE was not fully removed from the membrane, which could be due to the short time of incubation.

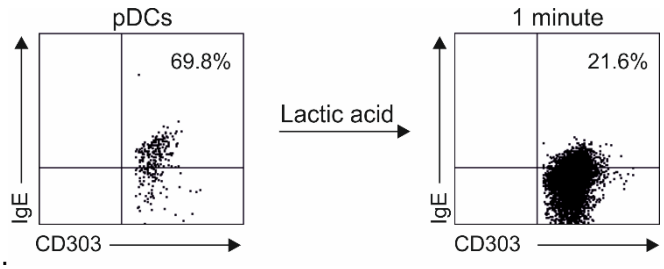


**Figure R.34. Lactic acid stripping of IgE$^+$ pDCs**. Representative example of the expression of IgE in pDCs purified from PBMCs from an atopic donor after incubation with lactic acid for 1 minute.

As 1 minute of stripping did not detach all the IgE bound to pDCs, a 5 minutes stripping was tested. The efficiency of 5 minutes of lactic acid incubation was higher than the one observed for 1 minute in the previous example, reducing the IgE$^+$ pDCs from 47.1% to 10.6% (**Figure R.35A**). Besides, the viability of pDCs after a 5 minutes stripping with lactic acid was tested using eFluor 660 viability dye. pDCs purified from PBMCs treated during 5 minutes with lactic acid displayed a similar viability than freshly purified pDCs from PBMCs (75.5% vs 77.2% of viability, **Figure R.35B**).

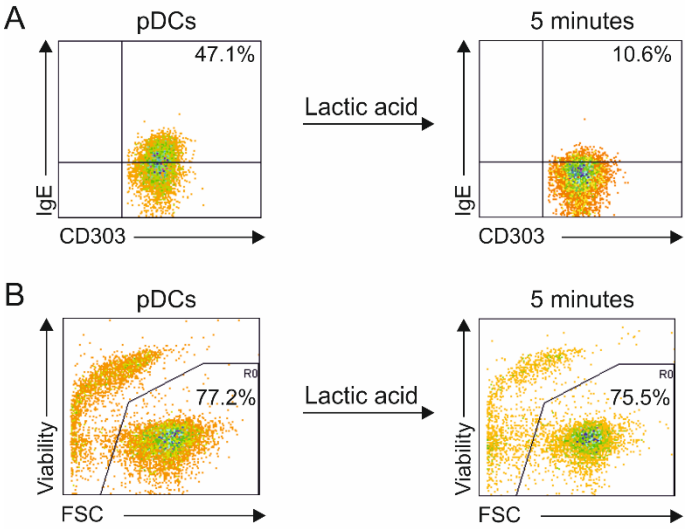


**Figure R.35. Lactic acid stripping of IgE$^+$ pDCs**. **A**, Representative example of the expression of IgE in pDCs purified from stripped PBMC with lactic acid for 5 minutes. **B**, Viability of the purified pDCs.

Despite these results, differences in the efficacy of the stripping have been observed depending on the initial percentage of IgE$^+$ pDCs (data not shown). When the initial percentage of IgE$^+$ pDCs from an atopic donor was very high, the efficiency of the stripping was lower than in the cases where there were less initial IgE$^+$ pDCs.

### *2.1. Optimization of pDC-stimulation conditions after IgE-lactic acid stripping*

After IgE stripping, we wanted to incubate pDCs with IgE or IgE previously incubated during 1 hour with ligelizumab or omalizumab with continuous stirring. To optimize the binding of exogenous IgE after the stripping, different concentrations of IgE were added to pDCs during 1 hour and the levels of IgE on the surface were analysed by flow cytometry. Around 25% of previously stripped pDCs incubated with 5 nM (1 µg/mL) of IgE bound IgE on the membrane without affecting the size and complexity of the cells (**Figure R.36**). Although pDCs incubation with higher doses of exogenous IgE induced a higher percentage of $IgE^+$ pDCs, cell viability was affected (**Figure R.36**). Therefore, 5 nM of IgE was selected for the rest of the experiments.

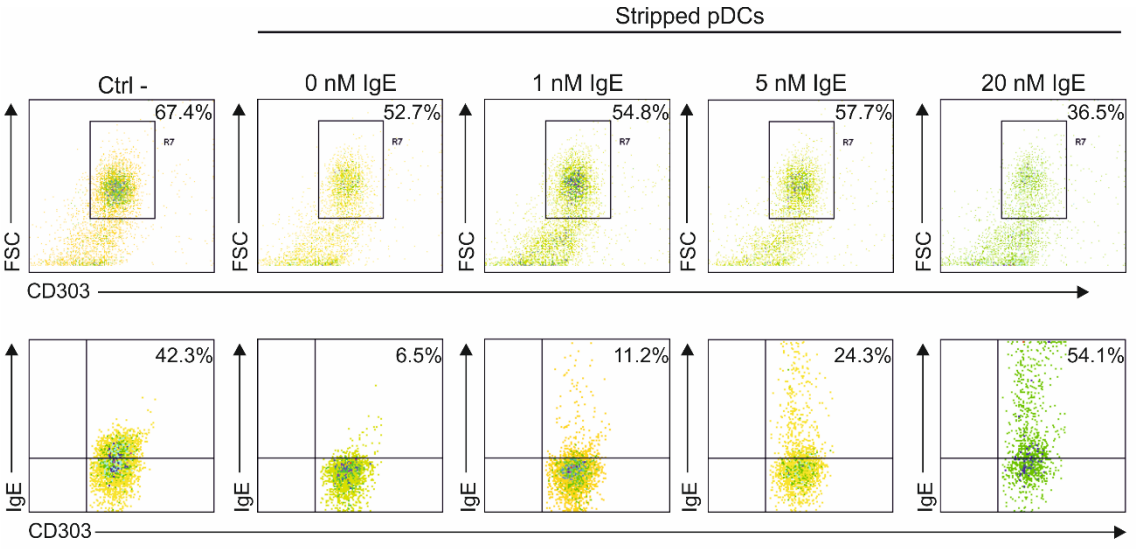


**Figure R.36. Binding of exogenous IgE to stripped-pDCs**. pDCs were stripped with lactic acid for 5 minutes. Later, pDCs were incubated with exogenous IgE at the indicated concentrations for 1 hour and the percentage of bound IgE was analysed by flow cytometry.

To compare the effects of omalizumab and ligelizumab in pDCs, after removal of IgE, pDCs were loaded with IgE alone or IgE pre-incubated with omalizumab or ligelizumab. Omalizumab binds free IgE at the high-affinity receptor-binding site. Once bound to the IgE, small complexes are formed. Typically, those complexes are trimers of approximately 490-530 kD or hexamers of approximately 1000 kD. The size of the complexes depends on the proportion of free IgE and omalizumab.[36] Therefore, for our experiments, different ratios IgE:Anti-IgE mAbs were tested. In all the ratios tested (1:0.5, 1:1, 1:2), ligelizumab blocked IgE-binding to pDCs more efficiently than omalizumab did (**Figure R.37**). As mention above, ligelizumab binds IgE with higher affinity than omalizumab. Therefore, it was expected that ligelizumab blocks the binding of free IgE to the surface of pDCs more efficiently than omalizumab.[101]

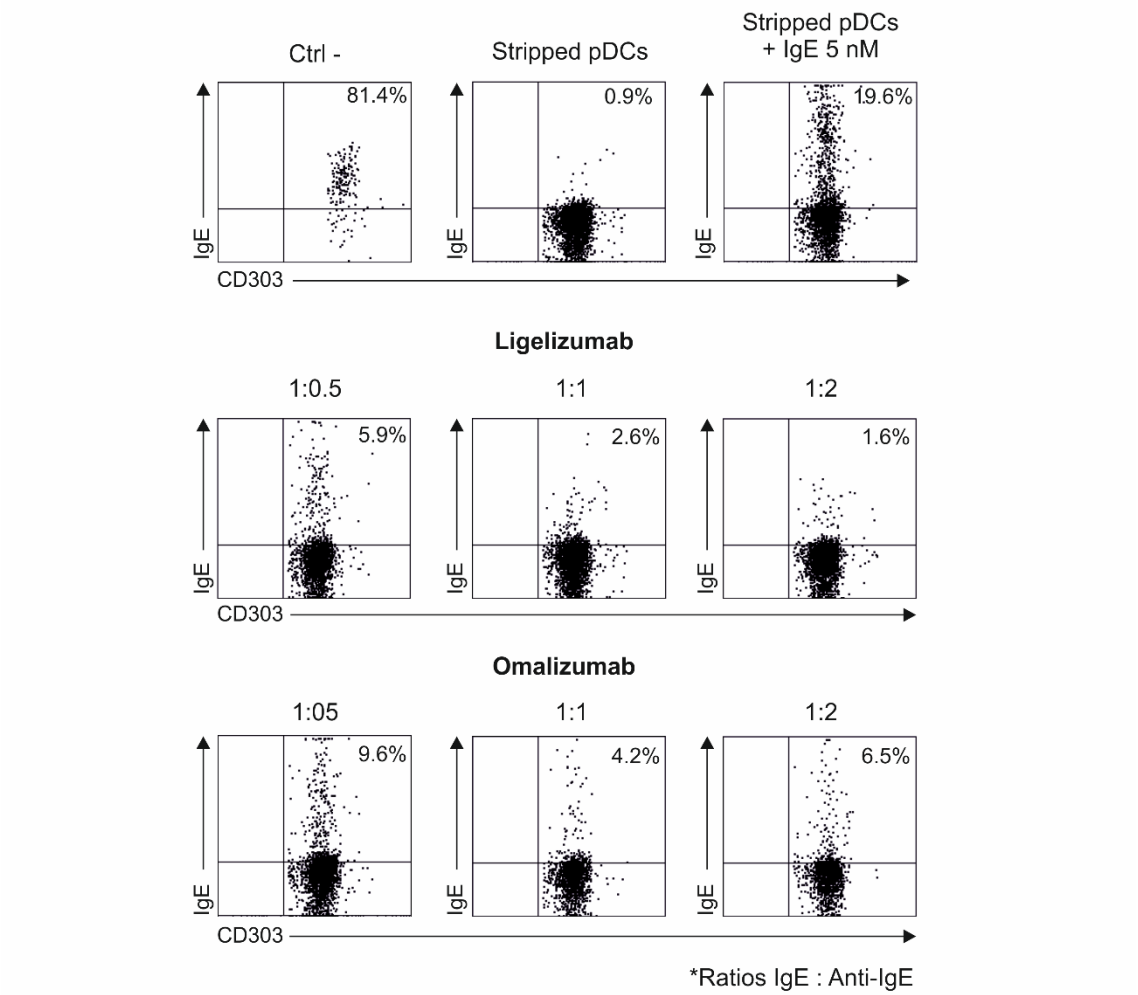


**Figure R.37. Optimization of the capacity of ligelizumab and omalizumab to sequester free IgE.** After lactic acid stripping, pDCs were incubated with IgE (5 nM, equivalent to 1 µg/mL) or with different ratios of IgE:Anti-IgE mAbs during 1 hour to study the proportion of IgE bound to FcεRI on the surface of the cells. The plots show a representative example.

Among the different ratios of IgE:Anti-IgE mAbs, the ratios 1:0.5 and 1:1 showed a significant difference between ligelizumab and omalizumab, with a higher capacity to capture free IgE by ligelizumab than omalizumab (**Figure R.38**).

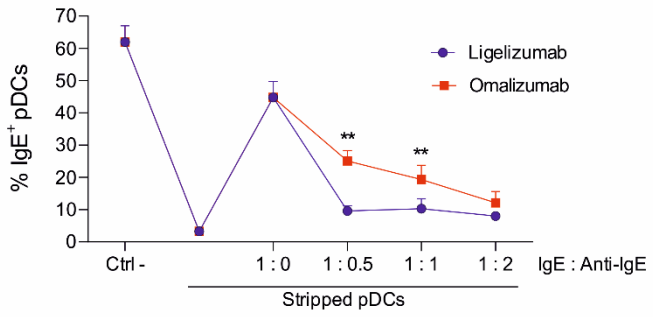


**Figure R.38. Optimization of free-IgE sequestered by ligelizumab and omalizumab.** After lactic acid stripping, pDCs were incubated with IgE or with different ratios of IgE:Anti-IgE mAbs during 1 hour to study the proportion of IgE bound to FcεRI on the surface of pDCs (n = 8-10). Values are given as mean ± SEM. ***P*<0.01 in Paired Student *t* test comparing multiple conditions.

The ratio 1:0.5 might well induce the generation of trimeric complexes of ligelizumab/omalizumab with IgE, in which one molecule of the mAb binds to two IgE molecules. For the ratio 1:1, the generated complexes could be hexamers, in which 3

molecules of ligelizumab/omalizumab bind to 3 IgE molecules (**Figure R.39**). For the next experiments, we selected the ratio 1:0.5, which displayed the highest difference between ligelizumab and omalizumab in terms of blocking capacity of exogenous IgE binding to FcεRI on pDCs.

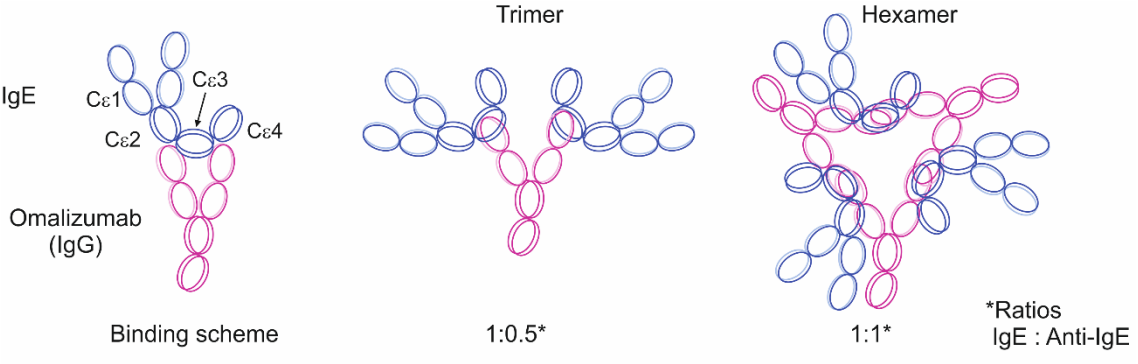


**Figure R.39. Structure of the generated complexes IgE:Anti-IgE mAbs.** Omalizumab and ligelizumab bind IgE through the Cε3 domain forming trimeric or hexameric complexes depending on their molecular ratios. Adapted from *Anti-IgE therapy: clinical utility and mechanistic insights. Curr Top Microbiol Immunol. 2015; 388:39-61.*

### *2.2. IgE-mediated FcεRI-crosslinking effects on cytokine production after IgE-lactic acid stripping*

Once all these experimental conditions were stablished, we wanted to analyse the influence of the presence of ligelizumab or omalizumab in the phenotype and functional features of pDCs. Therefore, we purified pDCs from PBMCs from atopic donors. With lactic acid treatment, we removed surface-IgE and pDCs were then incubated with exogenous IgE-alone (5 nM), IgE:ligelizumab (1:0.5) or IgE:omalizumab (1:0.5) for 1 hour. Later, cells were washed and IgE-crosslinking was induced with the rabbit anti-human IgE (CL, 10 µg/mL) for 18 hours. Then, cells were washed again and stimulated with TLR9-L for 18 hours to analyse their phenotype and cytokine production.

As expected, stimulation of pDCs with TLR9-L induced an increase in the production of IFN-α and TNF-α compared to unstimulated pDCs. IgE-crosslinking in the TLR9-L-activated pDCs induced a significant decrease in the production of IFN-α, which was significantly reverted by ligelizumab and omalizumab (**Figure R.40**). Remarkably, the capacity to revert IFN-α production after IgE-FcεRI crosslinking in TLR9-L-activated pDCs was significantly higher for ligelizumab than omalizumab, which can be related to the higher capacity of ligelizumab to block exogenous IgE binding to pDCs. However, under the assayed conditions, we did not detect significant changes in the production of TNF-α upon IgE-FcεRI crosslinking and, therefore, changes in the presence of ligelizumab or omalizumab were not detected either (**Figure R.40**).

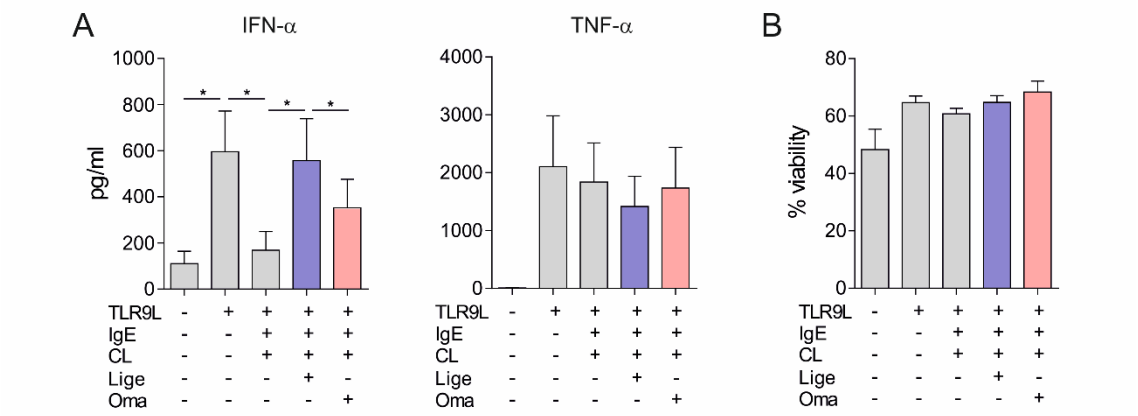


**Figure R.40. The presence of ligelizumab increases IFN-α production after IgE-FcεRI crosslinking on pDCs more efficiently than omalizumab. A,** IFN-α and TNF-α production after the indicated conditions, measured by ELISA (n = 6). **B**, Viability of pDCs (n = 4). Values are given as mean ± SEM. **P*<0.05 in Paired Student *t* test. *CL* IgE-FcεRI-crosslinker, *Lige* Ligelizumab, *Oma* Omalizumab.

### *2.3. IgE-mediated FcεRI-crosslinking effects on Treg induction after IgE-lactic acid stripping*

Next, we wanted to study the effects of the presence of ligelizumab and omalizumab in the capacity of pDCs to generate Treg cells. For that, we performed the same type of experiments described above. We cocultured pDCs stimulated under different conditions with allogeneic naïve $CD4^+$ T cells for 5 days (**Figure R.41**).

pDCs stimulated with TLR9-L induced the generation of more Treg cells than unstimulated pDCs. However, under the assayed conditions, we did not detect changes in the induction of Treg cells by TLR9-L-activated pDCs upon IgE-crosslinking and, therefore, changes in the presence of ligelizumab or omalizumab were not detected either (**Figure R.41)**.

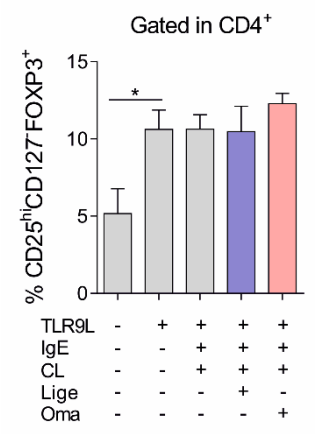


**Figure R.41. There are no changes in the induction of Treg cells with this protocol.** $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells generated after 5 days by stripped pDCs from atopic donors treated with the indicated conditions (n = 5). Values are given as mean ± SEM. **P*<0.05 in Paired Student *t* test. *CL* IgE-FcεRI-crosslinker, *Lige* Ligelizumab, *Oma* Omalizumab.

We hypothesized that the absence of changes in Treg cells generation after IgE-crosslinking might be due to unknown effects of acid stripping on the properties of pDCs.

Previous studies have reported that acid stripping of surface IgE antibodies bound to FcεRI might be unsuitable for the functional assays that require long-term culture of basophils and entire removal of surface IgE.[201] Acid treatments can be used for the removal of IgE from FcεRI for the immediate characterization of the cells but they are not suitable for functional assays. Therefore, we sought to investigate other approaches for IgE stripping on pDCs.

## 3. Stripping of IgE with DARPin bi53-79

DARPins (designed ankyrin repeat proteins) are designed proteins derived from natural ankyrin repeat proteins composed of several repeat subunits, each representing a binding domain. The assembled protein consists of two capping repeats intervened by two or more binding modules that are randomly modified on the surface. DARPins of different repeat numbers have been constructed and selected for different targets.[202, 203] In the last years, bispecific DARPins which efficiently antagonize the IgE receptor have been characterized in the field of allergy.[204, 205]

The bispecific DARPin bi53-79 protein recognizes FcγRIIB and FcεRI-bound IgE on the surface of the allergic effector cells.[206] FcγRIIB (CD32b) is an inhibitory receptor expressed on different immune cells including basophils and mast cells which binds IgG with low affinity.[207] There is evidence that FcγRIIB is implicated in negative regulation of immediate hypersensitivity reactions and that co-aggregation of FcγRIIB and FcεRI on the surface of allergic effector cells is a promising approach to inhibit allergen-dependent hypersensitivity reactions.[208, 209] It has been described that this bispecific DARPin has the ability to co-ligate FcγRIIB and FcεRI-bound IgE on allergic effector cells.[206] Besides, DARPin bi53-79 efficiently dissociates IgE-FcεRI complexes greater than other bispecific DARPins and omalizumab.[210] Therefore, we decided to use the described DARPin to perform the stripping of IgE in atopic pDCs. The DARPin was kindly provided by Alexander Eggel from Bern University, Switzerland, under a proper material transfer agreement between Universities.

Firstly, we optimized the concentration and time of incubation with the DARPin because it had never been used in pDCs. For that, we checked 30 minutes and 1 hour of incubation with 1, 0.5, and 0.1 µM of DARPin. The purified pDCs from an atopic donor used for this experiment displayed an 82.5% of $IgE^+$ pDCs. Most of the IgE was bound to the high affinity receptor FcεRIα (**Figure R.42A**). The purified pDCs were treated with DARPin at the different conditions, and then incubated with 5 nM of IgE for 1 hour to test how they bind exogenous IgE after the removal of the endogenous IgE.

We observed that the best conditions for the dissociation of IgE-FcεRI complexes was a concentration of 1 µM during 1 hour of incubation, after which the remaining percentage of $IgE^+$ pDCs was just around 5%. When the concentration of DARPin was lower, or the time of incubation was shorter, the remaining percentage of $IgE^+$ pDCs was higher (**Figure R.42B**). Afterwards, the stripped pDCs were able to bind exogenous IgE, returning to slight lower percentages of $IgE^+$ pDCs than the initials when the endogenous IgE had been removed properly (**Figure R.42B**). Therefore, we chose 1 hour of incubation with 1 µM of DARPin as the new stripping conditions for the experiments.

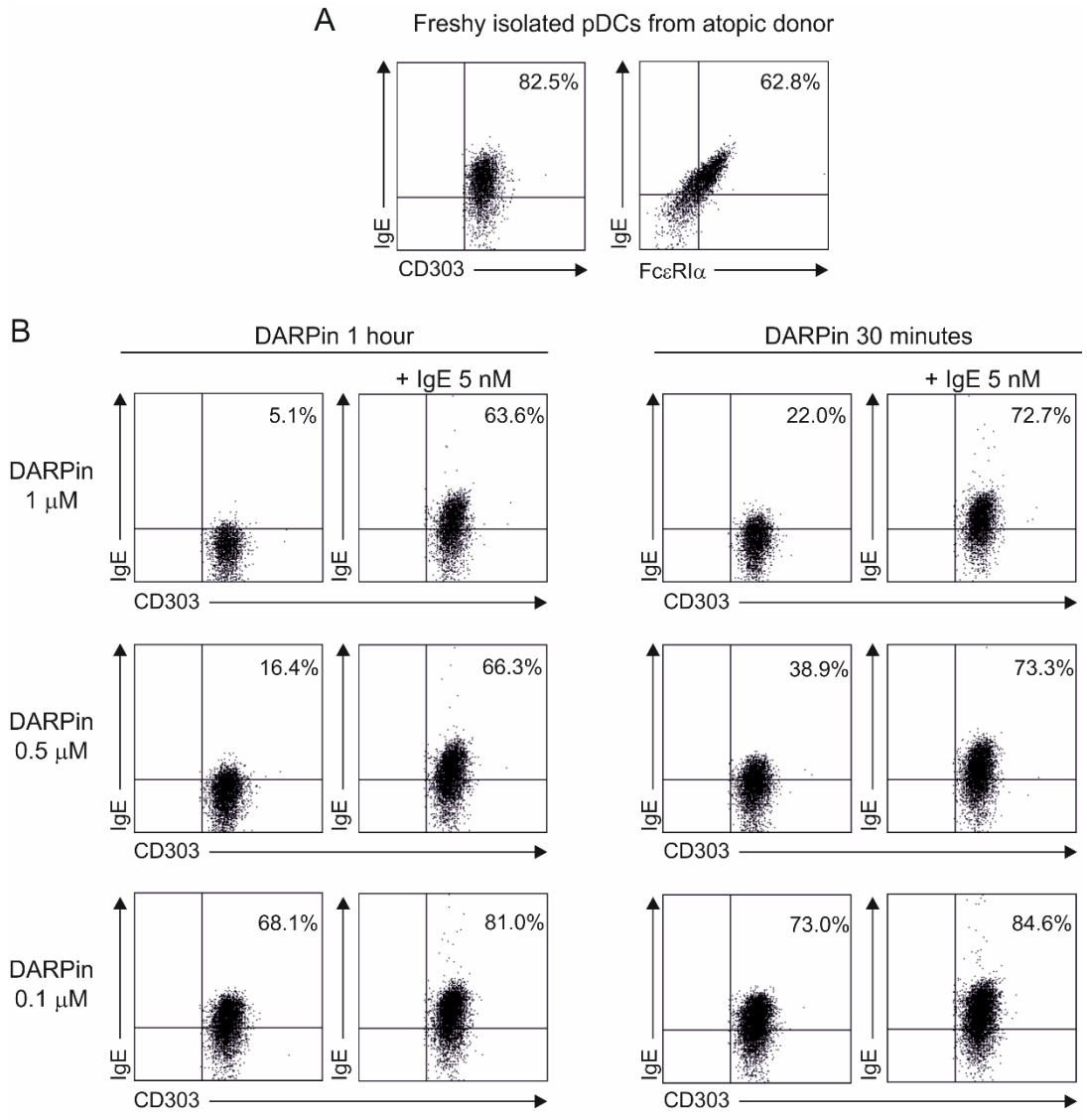


**Figure R.42. Optimization of IgE-stripping on the surface of pDCs with DARPin bi53-79. A**, IgE and FcεRIα expression in purified pDCs from an atopic donor. **B**, Percentage of IgE bound to the surface of pDCs after the treatment with DARPin bi53-79 at the indicated conditions. After DARPin treatment, pDCs were incubated for 1 hour with 5 nM of IgE. After washing the cells, the percentage of bound-IgE was quantified by flow cytometry.

We wanted to check the capacity of ligelizumab and omalizumab to prevent the binding of exogenously added free IgE to the IgE-stripped pDCs. Therefore, after

DARPin treatment, pDCs were incubated for 1 hour with exogenous IgE (at 5 nM), IgE:ligelizumab, or IgE:omalizumab (at 1:0.5 ratios). As described above, freshly purified pDCs displayed IgE on the surface, mainly bound to FcεRI. After DARPin treatment, almost all the IgE was removed from the surface of pDCs (**Figure R.43**). It is known that the presence of IgE bound to FcεRI is essential for the stabilization of the receptor.[211] However, we have observed that after DARPin treatment, the α chain of the FcεRI was still expressed on the membrane of pDCs (**Figure R.43**). After exogenous IgE addition, pDCs were able to bind the IgE to the FcεRI, rendering a similar profile to that observed for freshly pDCs. However, when the IgE had been pre-incubated with ligelizumab or omalizumab, there was a significant reduction in the binding of IgE to FcεRI on pDCs. As we had seen after acid lactic treatment, ligelizumab blocked the IgE-binding to the pDCs more efficiently than omalizumab did (**Figure R.43**).

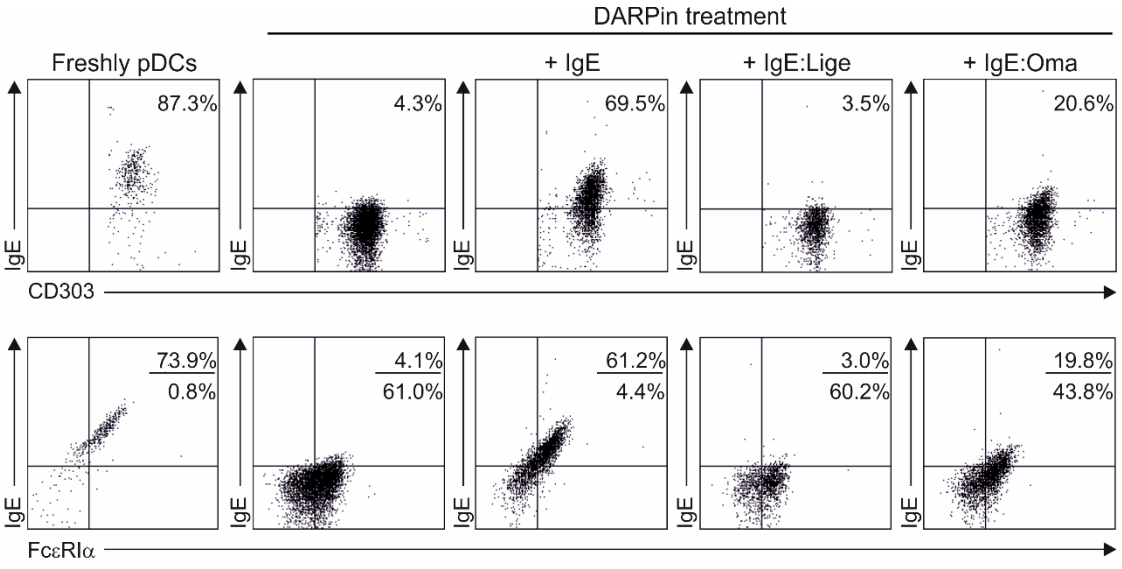


**Figure R.43. Ligelizumab blocked the IgE-binding to the pDCs more efficiently than omalizumab.** pDCs were purified from PBMCs from an atopic donor and incubated for 1 hour with 1 μM of DARPin. Then, cells were washed and incubated 1 hour with IgE 5 nM, IgE:ligelizumab, or IgE:omalizumab (1:0.5) to analyse the expression of IgE and FcεRIα on the surface of the cells by flow cytometry.

### *3.1. Effect of ligelizumab and omalizumab on cytokine production by pDCs after DARPin IgE-stripping*

To analyse the functional implications of the bound-exogenous IgE on the pDCs surface under the different treatment conditions (upon DARPin IgE-stripping and subsequent incubation with IgE alone, IgE:ligelizumab, or IgE:omalizumab), we crosslinked the exogenous bound-IgE with CL, washed the cells and stimulated them with TLR9-L to quantify cytokine production.

pDCs responded correctly to TLR9-L-stimulation by producing high amounts of IFN-α and TNF-α. After IgE-mediated FcεRI-crosslinking on pDCs, there was a significant reduction on the production of IFN-α, which was slightly reverted by ligelizumab but not by omalizumab (**Figure R.44**). Regarding TNF-α production, IgE-crosslinking on pDCs induced a slight increase in its production, with a small reversion when IgE had been previously incubated to ligelizumab and omalizumab (**Figure R.44**).

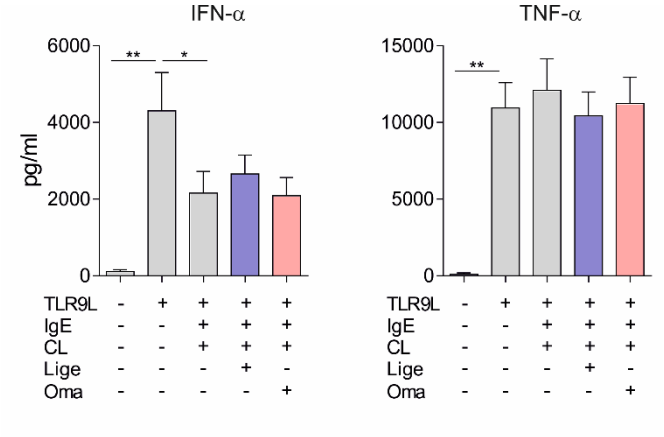


**Figure R.44. Ligelizumab slightly reverts the effects of IgE-mediated FcεRI-crosslinking.** IFN-α and TNF-α cytokines measured by ELISA (n = 6). Values are given as the mean ± SEM. ***P*<0.01, **P*<0.05 in Paired Student *t* test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker, *Lige* Ligelizumab, *Oma* Omalizumab.

### *3.2. Effect of ligelizumab and omalizumab on pDC capacity to generate Treg cells after DARPin IgE-stripping*

To analyse the induction of Treg cells by pDCs after IgE-FcεRI crosslinking and the influence of the blocking of IgE-binding to the surface of the cells by ligelizumab or omalizumab, after DARPin-dependent IgE stripping, incubation with IgE alone, IgE:ligelizumab, or IgE:omalizumab, and stimulation with CL for IgE-FcεRI crosslinking, we treated pDCs with TLR9-L and cocultured them for 5 days with allogeneic naïve $CD4^+$ T cells in a 1:5 ratio (pDCs: naïve T cell ratio). To analyse Treg cells, we have measured the production of IL-10 in the coculture supernatant as well as the generation of $CD4^+CD25^{high}CD127^-FOXP3^+$ cells by flow cytometry.

IgE-stripped TLR9-L-stimulated pDCs from atopic donors induce significantly more IL-10-producing T cells than IgE-stripped unstimulated pDCs. IgE-mediated FcεRI-crosslinking reduced the production of IL-10 by T cells cocultured with TLR9-L-stimulated pDCs (**Figure R.45A**), as previously described.[191] When pDCs were treated with IgE previously incubated with ligelizumab but not with omalizumab, the production of IL-10 by T cells was slightly increased and restored (**Figure R.45A**).

Although the influence of IgE-FcεRI crosslinking in TLR9-L-activated pDCs with this protocol only showed a slight tendency of IL-10-production by cocultured T cells, we have observed that it impaired the capacity of TLR9-L-stimulated pDCs to induce Treg

cells. Interestingly, that impaired capacity is reverted in the presence of ligelizumab or omalizumab, without significant differences between the two anti-IgE mAbs (**Figure R.45B**).

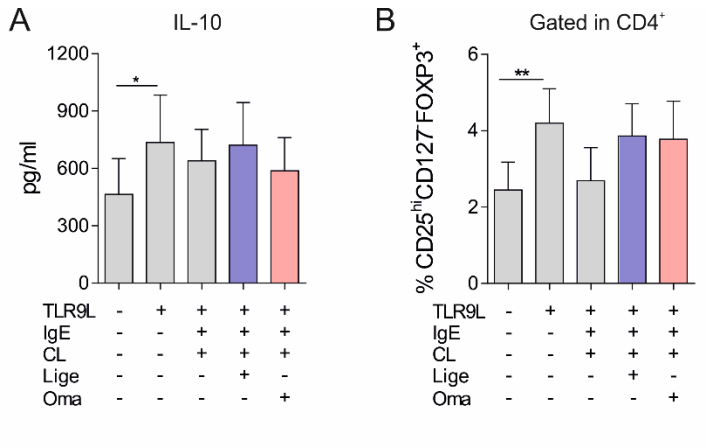


**Figure R.45. Ligelizumab and omalizumab restore the capacity of pDCs to induce Treg cells. A**, IL-10 cytokine measured in coculture supernatant by ELISA (n = 7). **B**, $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells generated after 5 days by DARPin stripped-pDCs from atopic donors treated with the indicated conditions (gating on lymphocytes, n = 6). Values are given as the mean ± SEM. **P<0.01 in Paired Student *t* test comparing multiple conditions. *CL* IgE-FcεRI-crosslinker, *Lige* Ligelizumab, *Oma* Omalizumab.

Our experiments corroborated the results previously published for omalizumab in this new model from IgE-stripped pDCs from atopic donors recharged with exogenous IgE. The IgE-mediated FcεRI-crosslinking impairs the ability of TLR9-L-activated pDCs to induce the generation of Treg cells, which is restored by omalizumab. Herein, we have observed also a restoration of Treg cell generation by ligelizumab, without significant differences between the two anti-IgE mAbs.

## 4. Treatment of pDCs with specific IgE

To ensure a better specificity in our experimental setting, we used specific IgE in order to crosslink just the exogenous IgE in an antigen-specific manner. For that, we used chimeric human 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP)-specific IgE JW8 and NIP-BSA (bovine serum albumin) conjugate to crosslink IgE-FcεRI.

Firstly, we tested the optimal concentration of IgE-NIP to be loaded on pDCs. pDCs were IgE-stripped with DARPin 1 µM for 1 hour, washed and incubated with 2.5, 5, and 10 nM of IgE-NIP to analyse the bound-IgE to the surface of pDCs. pDCs treated with 10 nM of IgE-NIP displayed the highest percentage of bound-IgE (**Figure R.46**). Therefore, we selected this concentration for further experiments.

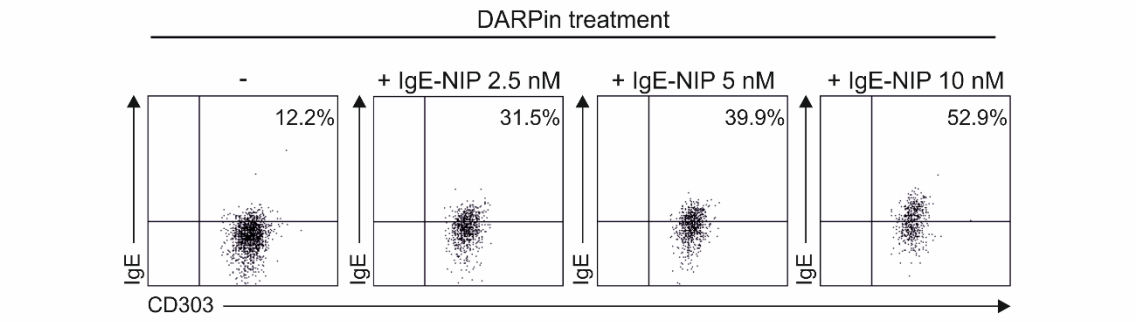


**Figure R.46. Optimization of stripped-pDCs binding of NIP-specific IgE.** pDCs were IgE-stripped with DARPin for 1 hour. Later, cells were washed and incubated with NIP-specific IgE at the indicated concentrations for 1 hour, and the percentage of bound IgE was analysed by flow cytometry. *IgE-NIP* 4-hydroxy-3-iodo-5-nitrophenylacetyl-specific IgE.

### *4.1. Effect of ligelizumab and omalizumab on Treg cell induction by pDCs with NIP specific-IgE*

To analyse the generation of Treg cells, pDCs loaded with 10 nM of IgE-NIP were treated with 10 ng/mL of NIP-BSA to induce the IgE-FcεRI crosslinking. Then, cells were stimulated with TLR9-L and cocultured with allogeneic naïve $CD4^+$ T cells for 5 days at a 1:5 ratio (pDC: $CD4^+$ T cell ratio). We also observed that specific-IgE-FcεRI crosslinking in TLR9-L-activated pDCs impaired the capacity of pDCs stimulated with TLR9-L to induce Treg cell polarization. Interestingly, that impaired capacity was significantly reverted in the presence of ligelizumab and omalizumab. Although there was no significant difference between the two anti-IgE mAbs, we could observe a tendency of a higher reversion by omalizumab compared to ligelizumab (**Figure R.47**).

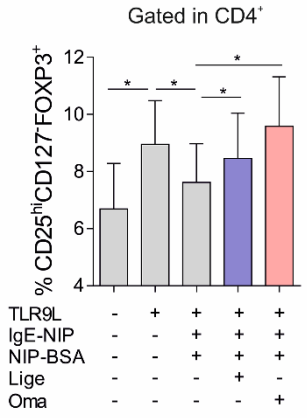


**Figure R.47. Both ligelizumab and omalizumab restore the capacity of pDCs to induce Treg cells using NIP-specific IgE.** $CD4^+CD25^{high}CD127^-FOXP3^+$ Treg cells generated after 5 days by DARPin stripped-pDCs from atopic donors treated with the indicated conditions (gating on lymphocytes, n = 6). Values are given as the mean ± SEM. **P<0.01 in paired t-test comparing multiple conditions. *IgE-NIP* 4-hydroxy-3-iodo-5-nitrophenylacetyl-specific IgE, *NIP-BSA* NIP-bovine serum albumin conjugate, *Lige* Ligelizumab, *Oma* Omalizumab.

In summary, our data provide novel insights into the capacity of ligelizumab to restore the ability of TLR9-L-activated pDCs to induce the generation of Treg cells. We have demonstrated that ligelizumab displays a higher capacity to block free IgE and

avoid exogenous binding to FcεRI on pDCs than omalizumab, likely due to its higher affinity for IgE. In contrast to omalizumab, ligelizumab is not able to remove the FcεRI-bound IgE on atopic pDCs due to the fact that this mAb recognizes distinct binding epitopes in IgE Cε3 domain than omalizumab. Our current results do not allow us to stablish whether there might be significant differences between the effect of ligelizumab and omalizumab in the generation of Treg cells by TLR9-L-stimulated pDCs. Further ongoing studies are required to obtain firm conclusions at this regard for these two anti-IgE mAbs.

# GLOBAL DISCUSSION

In this Doctoral Thesis, we provide novel insights into the molecular mechanisms involved in the mode of action of next generation AIT vaccines (allergoid-mannan conjugates) as well as anti-IgE biologicals (omalizumab and ligelizumab) as treatment approaches for different allergic diseases. The understanding of the immunological mechanisms underlying allergy and the processes involved in the induction of allergen tolerance are crucial for the development and improvement of strategies to treat and prevent allergic diseases.

The prevalence of allergy has dramatically increased worldwide over the last decades, generating a high socio-economic impact and reducing the quality of life of many patients, especially of those suffering from severe phenotypes.[6, 7] Nowadays, the only disease-modifying treatment with potential long-lasting curative capacity is AIT.[54] AIT was developed 110 years ago, and significant improvements have been done since then.[53, 62] Together with AIT, biologicals, such as mAbs, represent another therapeutic strategy that is revolutionizing the treatment of several allergic diseases. Different biologicals have proven to be efficacious for the treatment of allergic diseases.[94, 97] However, it has not been reported yet that biologicals prevent or modify the course of the allergic disease upon treatment discontinuation.[97] Although AIT and biologicals represent the most effective and advanced therapies used in clinics, they still display several important drawbacks in terms of security, low adherence, long duration, efficacy, side effects or high cost.[94, 212] Therefore, there is an urgent need to improve these treatments.

The disease-modifying effects of AIT are related to the immune modulation of both the innate and adaptive responses. At this regard, DCs play a key role in the induction of tolerance, representing the link between innate and adaptive immune system.[213] Importantly, sustained tolerance after AIT requires the generation of Treg and Breg cells as well as allergen-specific blocking antibodies.[25, 65] Here, we have demonstrated that allergoid-mannan conjugates represent a suitable approach to induce allergen tolerance in AIT. It had been previously described that polymerized allergoids conjugated to nonoxidized mannan are efficiently captured by hmoDCs via mannose receptor and DC-SIGN, increasing IL-10 production and PD-L1 expression, and promoting the generation of functional FOXP3$^{+}$ Treg cells both *in vitro* and *in vivo*. Moreover, this vaccine induces potent blocking IgG antibodies in rabbits and display *in vivo* hypoallergenicity.[60, 61, 86]

One of the main questions now in the field of AIT is the optimal formulation for the vaccines. Adjuvants are used to induce a faster, stronger, and long-lasting response

to the vaccine.[75] Although intensive research to develop novel adjuvants has been performed in recent years, aluminium hydroxide remains the most used adjuvant in AIT.[75, 78] Herein, we have demonstrated that alum significantly influences the tolerogenic response induced by allergoid-mannan conjugates in DCs. Alum decreases the expression of PD-L1 and IL-10, molecules involved in the generation of functional Treg cells, while increasing the expression of CD-83, OX40-L, and pro-inflammatory cytokines (IL-23 and IL-6) in PM-activated DCs. Alum promotes the generation of Th1, Th2, and Th17 cells and decreases the numbers and suppression capacity of FOXP3$^+$ Treg cells induced by PM. Therefore, although the presence of alum can be important for other vaccines that require stronger responses or a depot effect to prevent adverse reactions, in the case of allergoid-mannan conjugates this adjuvant should not be included in the vaccine formulation. In addition, our results significantly contribute to the better understanding of the molecular mechanisms of alum adjuvanticity in the context of allergy, which are not completely understood yet.[80] Here, we show for the first time that alum impairs Treg cells generation through the inhibition of mTOR activation, the shift in glycolysis metabolism and the reduction of ROS production by PM-activated DCs. Collectively, we demonstrate the importance of understanding the influence of adjuvants as alum in novel vaccine formulations as AIT.

Vaccine administration sites are usual places of monocyte recruitment,[214, 215] and there is strong evidence that monocyte differentiation into DCs or MØ is highly influenced by signals of the local tissue environment.[176, 216] It has been described in the last years that differentiation of monocytes in the presence of endogenous or synthetic molecules as IL-10, TGF-β, rapamycin, glucocorticoids, vitamin D3, or cannabinoids, among others, induce the generation of tolerogenic DCs.[163, 175, 189] Tol-DCs display a key role in the induction of tolerance to allergens through the maintenance of immune homeostasis, accomplished by the induction of Treg cells, the production of regulatory cytokines such as IL-10, and TGF-β, the decrease of T cell proliferation and the induction of T cell apoptosis.[213, 217] Here, we demonstrate for the first time that the presence of allergoid-mannan conjugates during the differentiation of monocytes from both nonatopic and allergic subjects into DCs yields tol-DCs (mannan-tolDCs) characterized by a lower cytokine response to LPS stimulation, a higher expression of the tolerogenic molecules *PD-L1*, *IDO*, *SOCS1*, *SOCS3* and *IL10*, and a higher capacity to induce functional FOXP3$^+$ Treg cells compared to conventional DCs. Different studies have demonstrated that the regulation of tol-DC's function is highly connected to their metabolic reprogramming.[152, 162] We have observed that mannan-tolDCs shift their metabolism from Warburg effect and lactate production towards a higher mitochondrial oxidative

phosphorylation compared to DCs differentiated in the absence of the vaccine. Accordingly, other studies have reported that tol-DCs display an increased expression of genes related to OXPHOS, especially of electron transport chain complexes II and IV.[161] Interestingly, here we have demonstrated that the tolerogenic phenotype of mannan-tolDCs from both nonatopic and allergic subjects is stable upon pro-inflammatory stimulation due to the epigenetic reprogramming. Mannan-tolDCs express more anti-inflammatory miRNA-146 and lower pro-inflammatory miRNA-155 than conventional DCs, and display histone modifications in genes that contribute to the observed profile. This study shed light into the capacity of allergoid-mannan conjugates to induce a tolerized innate memory state in human DCs, which might well pave the way for the design of new generation therapies and vaccines for allergy and other immune-mediated diseases.

Similarly, the generation of tolerogenic MØ is key for the efficacy of AIT. A strong Th2 cytokine microenvironment primes innate cells as MØ, which can polarize into a pro-allergic phenotype (triggered by IL-4 and IL-13). On the contrary, the phenotypes M2b and M2c are involved in immune regulation and tissue remodelling. After AIT, mucosal MØ together with other cellular subsets as Treg and Breg cells, produce IL-10 and influence the generation on IgG4 blocking antibodies.[218] Here, we have showed that allergoid-mannan conjugates imprint tolerogenic features also in human MØ. Polarization of THP1 macrophages in the presence of allergoid-mannan conjugates induces the generation of an IL-10-producing M2c profile and differentiation of M1-like MØ in the presence of the vaccine induces the shift into an M2-like profile characterized by IL-10 production and the expression of tolerogenic molecules (**Figure D.48**).

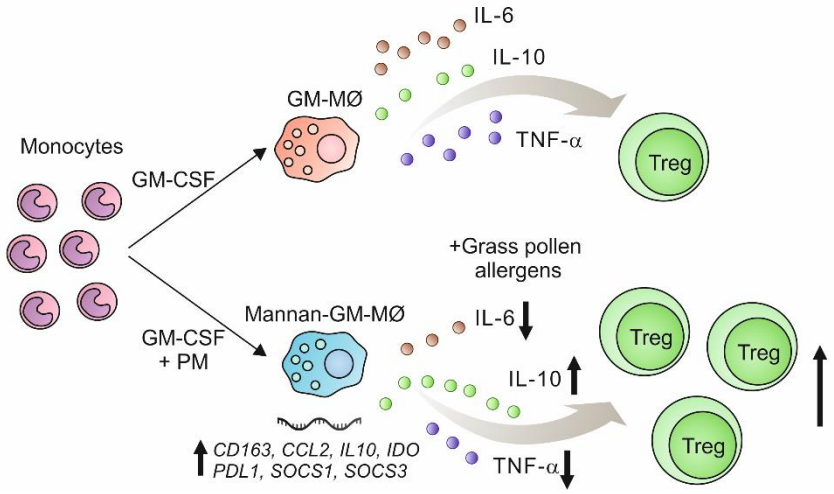


**Figure D.48. Allergoid-mannan conjugates (PM) imprint tolerogenic features in human macrophages.** Allergoid-mannan conjugates promote the generation of human IL-10-producing macrophages expressing more tolerogenic genes and inducing the generation of more Treg cells than conventional pro-inflammatory MØ.

These data uncovers the capacity of allergoid-mannan conjugates to also induce tolerogenic MØ that can contribute to the tolerogenic responses induced by this AIT vaccine. Remarkably, we have also demonstrated that Mannan-GM-MØ (MØ generated in the presence of the vaccine) from grass-pollen allergic patients loaded with native grass pollen allergens induce the generation of more $FOXP3^+$ Treg cells and IL-10 production than conventional GM-MØ (**Figure D.48**), favouring the generation of allergen-specific Treg cells essential for the efficacy of AIT.

As mentioned above, the most important novel therapeutic strategy for allergy besides AIT are biologicals, especially for the most severe patients.[47, 94, 97] Omalizumab was the first developed mAb for the treatment of severe allergic asthma specifically targeting IgE.[15] Recently, a next-generation high affinity anti-IgE mAb (ligelizumab) has been developed with the intention of improving the limitations associated to omalizumab.[101, 105] Omalizumab treatment increases the frequency of Treg cells in asthmatic children, which correlates with asthma control.[197] However, the mechanisms by which this anti-IgE mAb induces that tolerogenic phenotype remained unknown. Herein, we describe for the first time that IgE-FcεRI crosslinking on human pDCs from atopic donors impairs the capacity of pDCs to polarize $FOXP3^+$ Treg cells after TLR9-L stimulation. Omalizumab is able to detach the IgE bound to the surface of pDCs from atopic donors and therefore, it restores the ability of those pDCs to induce Treg cells. As shown in **Figure D.49,** IgE-FcεRI crosslinking in TLR9-L-activated pDCs impairs the production of IFN-α and IDO expression, whereas it increases the production of TNF-α. These three pathways are implicated in the capacity of pDCs to polarize Treg cells,[25] and they are restored by omalizumab. This is of paramount importance due to the fact that the induction and maintenance of Treg cells is essential for the tolerance against allergens, and could be a mechanism by which omalizumab could support that tolerance. However, more studies are needed to understand whether biologicals might induce a beneficial effect after ending the treatment or have positive disease-modifying effects to prevent the onset or the progression of the disease.[97]

Interestingly, recent studies have reported that omalizumab and ligelizumab recognize different binding epitopes of the IgE Cε3 domain, with just some overlap.[101] This can explain the inability of ligelizumab to remove the IgE bound to FcεRI on the surface of pDCs from atopic donors. However, due to its higher affinity for IgE compared to omalizumab, ligelizumab blocks the binding of exogenous IgE to FcεRI on pDCs more efficiently than omalizumab. Collectively, we have demonstrated here that ligelizumab is also able to restore the ability of TLR-9-stimulated pDCs from atopic donors to induce Treg cell polarization after IgE-FcεRI crosslinking, without significant differences with

omalizumab. Further studies are needed to compare in more detail these two anti-IgE mAbs.

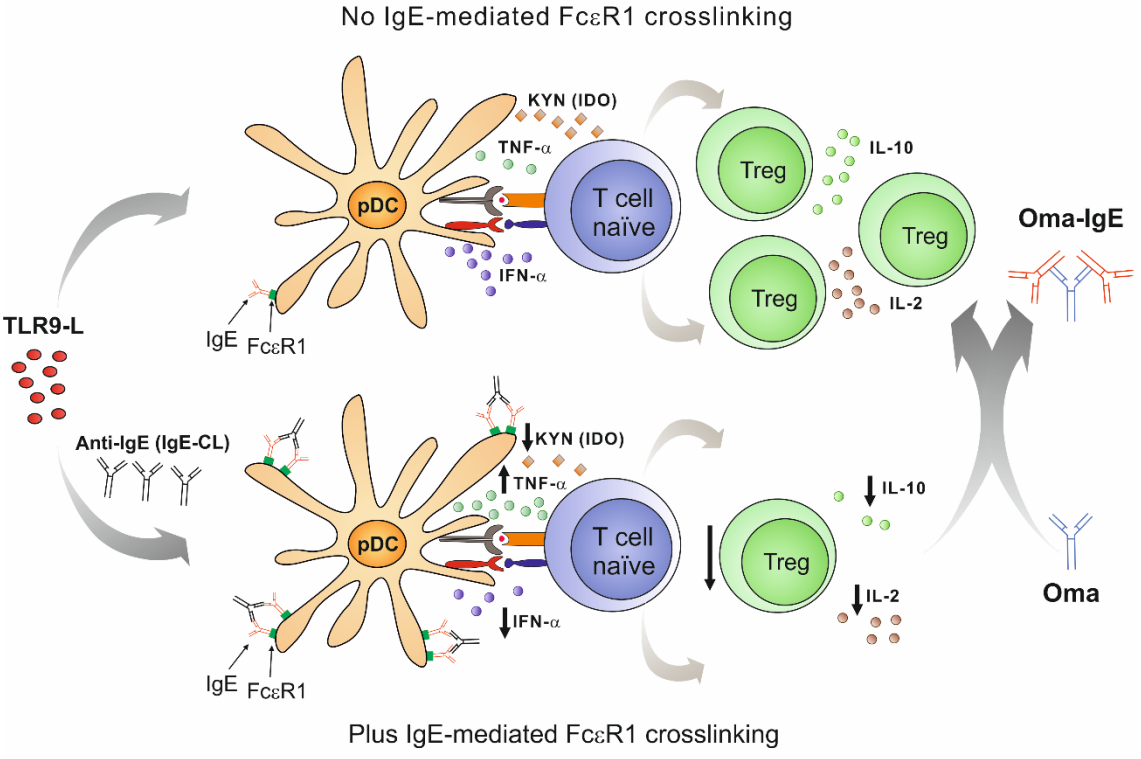


**Figure D.49. IgE-mediated FcεRI-crosslinking impairs the capacity of pDCs to induce Treg cells, which can be restored by omalizumab (oma).** Under TLR9-L stimulation, pDCs upregulate IDO and IFN-α secretion while decreasing TNF-α production. Consequently, Treg cell induction and increased IL-10 levels are induced. However, after IgE-mediated FcεRI-crosslinking, there is a decrease in the frequency of pDCs expressing IDO as well as IFN-α secretion while the production of TNF-α is increased. Consequently, there is an impairment of the capacity of pDCs to induce Treg cells, resulting also in reduced IL-10 secretion. Remarkably, oma removes membrane-bound IgE on pDCs, thus restoring their capacity to generate Treg cells after IgE-mediated FcεRI-crosslinking. *Oma* Omalizumab, *Kyn* Kynurenine.

In the last years several clinical trials have focussed on the possibility that biologicals could be used as add-on to AIT in order to decrease the onset and severity of adverse events during AIT or even to increase its efficacy.[62] For instance, in several double-bind, placebo-controlled clinical studies, omalizumab has been used as an adjunctive medication in patients with respiratory allergies receiving AIT, and demonstrating that the co-medication resulted in a significantly higher decrease of seasonal symptoms than AIT alone. Therefore, omalizumab could allow successful tolerance induction, especially in patients with high risk of systemic reactions to allergen administration in the context of AIT.[219]

In summary, we uncover previously unknown molecular mechanisms by which AIT and anti-IgE mAbs exert their beneficial effects acting on human DCs as advanced therapies for allergic disorders. The advances in the understanding of the mechanisms

implicated in the mode of action of these treatments will help us to identify novel biomarkers of efficacy and tolerance and might well pave the way for the design of better therapies in the future.

# CONCLUSIONS

The main conclusions of this Doctoral Thesis are:

## Block A

1. Alum impairs the tolerogenic properties promoted by allergoid-mannan conjugates by reducing the capacity of PM-activated DCs to generate functional FOXP3$^+$ Treg cells through a decrease in the expression of PD-L1 and IL-10 production, an inhibition of mTOR activation, shift in glycolysis metabolism and reduction of ROS production in human PM-activated DCs.

2. Subcutaneous immunization of mice with allergoid-mannan conjugates adsorbed to alum induces a shift to allergic responses and impairs the induction of splenic FOXP3$^+$ Treg cells, without affecting the induction of IgG-blocking antibodies.

3. Monocytes from nonatopic and allergic subjects differentiated into DCs in the presence of allergoid-mannan conjugates generate tolerogenic DCs (mannan-tolDCs) characterized by a shift in the glucose metabolism from Warburg effect and lactate production to mitochondrial OXPHOS compared to conventional DCs.

4. Allergoid-mannan conjugates imprint epigenetic reprogramming in the generated mannan-tolDCs from nonatopic and allergic donors involving specific histone marks, increased expression of the anti-inflammatory miRNA-146a/b and decreased pro-inflammatory miRNA-155, thus ensuring a stable tolerogenic phenotype even upon pro-inflammatory stimulation.

5. Allergoid-mannan conjugates promote the generation of human IL-10-producing macrophages with regulatory phenotypes (mannan-GM-MØ), which might well contribute to the tolerogenic responses induced by this novel AIT vaccine.

6. Mannan-GM-MØ from allergic patients loaded with native grass pollen allergens increase the generation of FOXP3$^+$ Treg cells compared to GM-MØ as well as their production of IL-10, indicating that mannan-GM-MØ are able to induce and expand allergen-specific Treg cells.

## Block B

1. IgE-FcεRI crosslinking in TLR9-L-activated pDCs impairs the generation of FOXP3$^+$ Treg cells and favours Th2 allergic profiles, which is restored by pre-treatment of pDCs with omalizumab.

2. IgE-FcεRI crosslinking in TLR9-L-activated pDCs impairs the production of IFN-α and IDO expression, whereas it increases TNF-α production. The levels of all

these molecules are partially or completely restored by omalizumab, suggesting that they might be potential candidates involved in the capacity of IgE-crosslinking to impair the generation of Treg cells by TLR9-L-activated pDCs.

3. The corticosteroid dexamethasone exhibits strong immunosuppressive properties and inhibits the capacity of both hmoDCs and pDCs to induce Treg cells after LPS- or TLR9-L-activation, respectively.

4. Ligelizumab displays the capacity to restore the ability of TLR9-L-activated pDCs to induce the generation of Treg cells after IgE-FcεRI crosslinking, which was similar to that observed for omalizumab.

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# APPENDIX

List of other publications:

- **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. doi: 10.1038/mi.2016.112. (Impact factor: 7.360; Q1 in immunology).

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- **Benito-Villalvilla C**, Soria I, Subiza JL, Palomares O. Novel vaccines targeting dendritic cells by coupling allergoids to mannan. *Allergo J Int*. 2018;27(8):256-262. doi: 10.1007/s40629-018-0069-8.

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- Martin-Cruz L, Sevilla-Ortega C, **Benito-Villalvilla C**, Diez-Rivero CM, Sanchez-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: 7.561; Q1 in immunology).

OPEN

# 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

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Recurrent urinary tract infections (RUTIs) are one of the most common bacterial infectious diseases, especially in women. Antibiotics remain the mainstay of treatment, but their overuse is associated with antibiotic-resistant infections and deleterious effects in the microbiota. Therefore, alternative approaches are fully demanded. Sublingual immunization with MV140 (Uromune), a polyvalent bacterial preparation (PBP) of whole heat-inactivated bacteria, demonstrated clinical efficacy for the treatment of RUTIs, but the involved immunological mechanisms remain unknown. Herein, we demonstrated that MV140 endorses human dendritic cells (DCs) with the capacity to generate Th1/Th17 and IL-10-producing T cells by mechanisms depending on spleen tyrosine kinase (Syk)- and myeloid differentiation primary response gene 88 (MyD88)-mediated pathways. MV140-induced activation of nuclear factor κB (NF-κB) and p38 in human DCs is essential for the generated Th1/Th17 and IL-10 immune responses whereas c-Jun N-terminal Kinase (JNK) and extracellular-signal regulated kinase (ERK) contribute to Th1 and IL-10 responses, respectively. Sublingual immunization of BALB/c mice with MV140 also induces potent systemic Th1/Th17 and IL-10 responses *in vivo*. We uncover immunological mechanisms underlying the way of action of MV140, which might well also contribute to understand the rational use of specific PBPs in other clinical conditions with potential high risk of recurrent infections.

## INTRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infectious diseases, especially in women.[1,2] Recurrent UTIs (RUTIs) are defined as three or more symptomatic UTIs during a year or two or more symptomatic UTIs within six months.[3] The pathogen most frequently isolated from patients with UTIs is *Escherichia coli* (80% of infections), followed by *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, *Proteus* spp and *Enterococcus spp*.[1,3,4] Antibiotics remain the mainstay of treatment for RUTIs,[1] but their overuse is associated with antibiotic-resistant infections and deleterious effects in the microbiota.[5–7] Therefore, there is an urgent need for the development of new alternative approaches to treat patients suffering from recurrent infections.[1]

Different types of mucosal bacterial vaccines have gained a lot of attention for the treatment of RUTIs over the last years. Bacterial vaccines may contain soluble antigens (bacterial components or lysates)[8,9] or inactivated whole bacteria from one or more species or strains.[10–12] Mucosal bacterial vaccines for RUTIs were initially administrated orally, but more recently the sublingual delivery route has been also assayed.[1,8,12]

The sublingual epithelium contains a dense network of dendritic cells (DCs) that play an essential role in linking innate and adaptive immune responses.[13,14] DCs are equipped with a

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Basic

European Journal of Immunology

Immunomodulation and immune therapies

Research Article

# Human dendritic cells activated with MV130 induce Th1, Th17 and IL-10 responses via RIPK2 and MyD88 signalling pathways

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**Recurrent respiratory tract infections (RRTIs) are the first leading cause of community- and nosocomial-acquired infections. Antibiotics remain the mainstay of treatment, enhancing the potential to develop antibiotic resistances. Therefore, the development of new alternative approaches to prevent and treat RRTIs is highly demanded. Daily sublingual administration of the whole heat-inactivated polybacterial preparation (PBP) MV130 significantly reduced the rate of respiratory infections in RRTIs patients, however, the immunological mechanisms of action remain unknown. Herein, we study the capacity of MV130 to immunomodulate the function of human dendritic cells (DCs) as a potential mechanism that contribute to the clinical benefits. We demonstrate that DCs from RRTIs patients and healthy controls display similar *ex vivo* immunological responses to MV130. By combining systems biology and functional immunological approaches we show that MV130 promotes the generation of Th1/Th17 responses via receptor-interacting serine/threonine-protein kinase-2 (RIPK2)- and myeloid-differentiation primary-response gene-88 (MyD88)-mediated signalling pathways under the control of IL-10. In vivo BALB/c mice sublingually immunized with MV130 display potent systemic Th1/Th17 and IL-10 responses against related and unrelated antigens. We elucidate immunological mechanisms underlying the potential way of action of MV130, which might help to design alternative treatments in other clinical conditions with high risk of recurrent infections.**

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ORIGINAL ARTICLE

Experimental Allergy and Immunology

WILEY Allergy

# Oral myeloid cells uptake allergoids coupled to mannan driving Th1/Treg responses upon sublingual delivery in mice

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## Abstract

**Background:** Polymerized allergoids coupled to nonoxidized mannan (PM-allergoids) may represent novel vaccines targeting dendritic cells (DCs). PM-allergoids are better captured by DCs than native allergens and favor Th1/Treg cell responses upon subcutaneous injection. Herein we have studied in mice the in vivo immunogenicity of PM-allergoids administered sublingually in comparison with native allergens.

**Methods:** Three immunization protocols (4-8 weeks long) were used in Balb/c mice. Serum antibody levels were tested by ELISA. Cell responses (proliferation, cytokines, and Tregs) were assayed by flow cytometry in spleen and lymph nodes (LNs). Allergen uptake was measured by flow cytometry in myeloid sublingual cells.

**Results:** A quick antibody response and higher IgG2a/IgE ratio were observed with PM-allergoids. Moreover, stronger specific proliferative responses were seen in both submandibular LNs and spleen cells assayed in vitro. This was accompanied by a higher IFNγ/IL-4 ratio with a quick IL-10 production by submandibular LN cells. An increase in $CD4^+CD25^{high}FOXP3^+$ Treg cells was detected in LNs and spleen of mice treated with PM-allergoids. These allergoids were better captured than native allergens by antigen-presenting ($CD45^+MHC\text{-}II^+$) cells obtained from the sublingual mucosa, including DCs ($CD11b^+$) and macrophages ($CD64^+$). Importantly, all the differential effects induced by PM-allergoids were abolished when using oxidized instead of nonoxidized PM-allergoids.

**Conclusion:** Our results demonstrate for the first time that PM-allergoids administered through the sublingual route promote the generation of Th1 and $FOXP3^+$ Treg cells in a greater extent than native allergens by mechanisms that might well involve their better uptake by oral antigen-presenting cells.

KEYWORDS
allergoid, glycoconjugate, immunotherapy, mannan, sublingual

**Abbreviations:** DC, dendritic cell; FOXP3, forkhead box P3; LN, lymph node; N, native allergen; PBS, phosphate-buffered saline; PE, phycoerythrin; PM-OX, oxidized form of PM; PM, polymerized allergoid coupled to nonoxidized mannan; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; Treg, regulatory T.

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review

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CrossMark

# Novel vaccines targeting dendritic cells by coupling allergoids to mannan

**Cristina Benito-Villalvilla · Irene Soria · José Luis Subiza · Oscar Palomares**

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**Abstract** Allergen-specific immunotherapy (AIT) is the single disease-modifying treatment for allergy. Clinical trials show AIT to be safe and effective for many patients; however, it still faces problems related to efficacy, safety, long treatment duration and low patient adherence. There has been intensive research to develop alternative strategies, including novel administration routes, adjuvants or hypoallergenic molecules. Promising results are reported for some of them, but clinical progress is still moderate. Allergoids conjugated to nonoxidized mannan from *Saccharomyces cerevisiae* have emerged as a novel concept of vaccine targeting dendritic cells (DCs). Preclinical human and animal models demonstrated that allergoids conjugated to mannan enhance allergen uptake, promote healthy responses to allergens by inducing Th1 and T regulatory (Treg) cells, and show clinical efficacy in veterinary medicine. Dose-finding phase II clinical trials in humans are currently ongoing. We review the current stage of allergoids conjugated to mannan as next generation vaccines for AIT.

**Keywords** Allergen-specific immunotherapy · Immune tolerance · Allergen vaccines · Allergoids conjugated to mannan · Dendritic cells · Regulatory T cells

## Abbreviations

| | |
|---|---|
| AIT | Allergen-specific immunotherapy |
| CLR | C-type lectin receptor |
| DC | Dendritic cell |
| FOXP3 | Forkhead box P3 |
| hmoDCs | Human monocyte-derived dendritic cells |
| mDCs | Myeloid dendritic cells |
| MPL | Monophosphoryl lipid A |
| N | Native allergen extracts |
| ODN | Oligodeoxynucleotide |
| P | Polymerized allergoids |
| pDCs | Plasmacytoid dendritic cells |
| PD-L1 | Programmed death-ligand 1 |
| PM | Polymerized allergoids conjugated to mannan |
| TLR | Toll-like receptor |
| Treg | Regulatory T cells |
| VLP | Viral like particle |

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## Introduction

Allergen-specific immunotherapy (AIT) is the only potential curative treatment for allergic diseases [1, 2]. AIT is based on the administration of high doses of the causative allergens to induce a state of permanent tolerance after treatment discontinuation. Currently, AIT is administered either subcutaneously or sublingually to restore healthy immune responses to allergens [3, 4]. Although many clinical trials and real-life experience show AIT as a safe and effective treatment for many allergic patients, it still faces important drawbacks. AIT remains underused mostly due to scattered availability of regimes and/or products for application, a lack of awareness, limited access to specialist care or the reimbursement policy [2, 5]. In addition, patient adherence is low, possibly due to the long duration and the large number of administrations needed to reach clinical efficacy [1]. Therefore, there is an urgent need to develop safer and more effective allergen vaccines that might help to overcome such drawbacks. In this regard, novel adjuvants or



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POSITION PAPER

WILEY Allergy

# Comparing biologicals and small molecule drug therapies for chronic respiratory diseases: An EAACI Taskforce on Immunopharmacology position paper

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**Abbreviations:** 5-LO, 5-Lipoxygenase; ACO, Asthma-COPD overlap; ADCC, Antibody-dependent cell-mediated cytotoxicity; AE, Adverse event; ALOX5, Arachidonate 5-Lipoxygenase; BBB, Blood-brain barrier; CCR2, C-C chemokine receptor type 2; CDC, Complement-dependent cytotoxicity; CD, Cluster of differentiation; c-KIT, CD117/stem cell growth factor receptor/ tyrosine-protein kinase; CNS, Central nervous system; COPD, Chronic obstructive pulmonary disease; COX, Cyclooxygenase; CRTH2= DP2, Chemoattractant receptor-homologous molecule expressed on T helper type 2 cells; CXCL2, Chemokine (C-X-C motif) ligand 2; CXCR2, C-X-C motif chemokine receptor 2/interleukin-8 receptor; CysLT, Cysteinyl leukotriene; CysLTRI, cysteinyl leukotriene receptor 1; DNA, Deoxyribonucleic acid; DP1, Prostaglandin D2 receptor 1; DP2, Prostaglandin D2 receptor 2; EGFR, Epidermal growth factor receptor; EMA, European Medicines Agency; FDA, Food and Drug Administration; FeNO, Fractional expired nitric oxide; FEV1, Forced expiratory volume in one second; GATA3, GATA binding protein 3; GINA, Global INitiative for Asthma; GM-CSF, Granulocyte-macrophage colony-stimulating factor; GOLD, Global initiative for chronic Obstructive Lung Disease; Gq, Guanine nucleotide-binding G protein subunit; GR, Glucocorticoid receptor; H1, Histamine receptor 1; IFN, Interferon; IgE, Immunoglobulin E; IL-4Rα, Interleukin-4 receptor alpha; IL-5Rα, Interlueukin-5 receptor α; ILC 1/2/3, Innate lymphoid cells group 1/2/3; IL, Interleukin; JAK, Janus kinase; LABA, Long-acting beta-agonists; LAMA, Long-acting muscarinic agonists; LTRA, leukotriene receptor antagonist; mAbs, Monoclonal antibodies; MAPK, Mitogen-activated protein kinase; MMP, Matrix metalloprotease; MR, Muscarinic receptor; p110δ, Phosphoinositide 3-kinase PI3K delta catalytic subunit delta; PDE, Phosphodiesterase; PGD2, Prostaglandin D2; PI3K, Phosphoinositide 3-kinase; PTGIR, Prostaglandin I2 receptor; SABA, Short acting beta-agonist; SAMA, Short acting muscarinic agonist; SMD, Small molecule drug; STAT, Signal transducer and activator of transcription protein; Th17, T helper cells that produce interleukin-17; Th1, T helper 1; Th2, T helper 2; Th9, T helper cells that produce interleukin-9; TNFα, Tumor necrosis factor alpha; TSLP, Thymic stromal lymphopoietin; TBXA2, Tromboxane receptor A2; ULABA, Ultra-long-acting beta-agonists; ULAMA, ultra-long-acting muscarinic agonists; WHO, World Health Organization.

[†]The Task Force of Immunopharmacology (TIPCO) within the Immunology Section of EAACI was established in 2017 to connect scientist and clinicians with the different scientific backgrounds—physicians and basic scientists, pharmacologists, computational biologists—with the task of examining recent breakthroughs on basic mechanisms of immune regulation and review their application in current, upcoming, and paradigm-shifting therapeutic approaches for allergy and clinical immunology-related diseases. The different topics for this first position paper, based on comparison of biologicals and small molecule drug therapeutic approaches, were assigned and drafted by authors' subgroups. They were further discussed, developed, and compiled during a meeting in Salerno (February 24-25, 2018). The position paper draft was thereafter recirculated and critically appraised until the final version was approved by all Task Force Members.

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EAACI POSITION PAPER

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# Immune modulation via T regulatory cell enhancement: Disease-modifying therapies for autoimmunity and their potential for chronic allergic and inflammatory diseases—An EAACI position paper of the Task Force on Immunopharmacology (TIPCO)

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**Abbreviations:** Ab, antibody; ACT, adoptive cell therapies; AD, atopic dermatitis; Ag, antigen; AMPK, AMP-activated protein kinase; APC, antigen-presenting cell; BAR, B cell–targeting Ab receptor; BCR, B-cell receptor; CAAR, chimeric autoantigen receptor; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; COPD, chronic obstructive pulmonary disease; CRISPR, clustered regularly interspaced short palindromic repeats; darTreg, donor-alloantigen-reactive Treg; Dsg3, desmoglein 3; EAE, experimental autoimmune encephalomyelitis; EMA, European Medicines Agency; FCεRI, high-affinity IgE receptor; FDA, United States Food and Drug Administration; FoxP3, forkhead box P3; GATA3, GATA binding protein 3; GMP, good manufacturing practice; GVHD, graft-versus-host disease; IFN, interferon; IL, interleukin; IL-2R, interleukin 2 receptor; iTr1, induced Tr1 cell; iTreg, in vitro-generated Treg; LAP, latency-associated peptide; mIgE, transmembrane form of IgE; mTOR, mammalian target of rapamycin; OIT, oral immunotherapy; PC, plasma cells; pTreg, peripherally induced Treg; PV, pemphigus vulgaris; RA, rheumatoid arthritis; scFv, single-chain variable fragment; SLE, systemic lupus erythematosus; SMD, small-molecule drug; T1D, type 1 diabetes; Tconv, conventional T cell (non-Treg); TCR, T-cell receptor; TGF-β, transforming growth factor β; Th1, T helper 1 cell; Th17, T helper 17 cell; Th2, T helper 2 cell; TIPCO, Task Force of Immunopharmacology; TMD, transmembrane domain; Tr1, type 1 regulatory cell; Treg, regulatory T cell; tTreg, thymus-derived Treg; UniCAR, universal CAR.

The Task Force of Immunopharmacology (TIPCO) within the Basic and Clinical Immunology Section of EAACI was established in 2017 to connect scientists and clinicians with different scientific backgrounds—physicians and basic scientists, pharmacologists, and computational biologists—with the task of examining recent breakthroughs on basic mechanisms of immune regulation and review their application in current, upcoming, and paradigm-shifting nonbiological therapeutic approaches for allergy and clinical immunology-related diseases. The topic for this second position paper was chosen unanimously, and specific parts were drafted by authors' subgroups. The first draft was compiled by first and corresponding authors and discussed in a general TF meeting in Vienna (March 29-30, 2019). The resulting draft was thereafter recirculated and critically appraised until the final version was approved by all Task Force members.

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

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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