UNIVERSIDAD COMPLUTENSE DE MADRID
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TESIS DOCTORAL

Estudio de la patogenia de la inmunodeficiencia de Jak1

Investigating the pathogenesis of Jaki immunodeficiency

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Vanessa Daza Cajigal

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Madrid

UNIVERSIDAD COMPLUTENSE DE MADRID

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DOCTORADO EN INVESTIGACIÓN BIOMÉDICA

DPTO. MICROBIOLOGÍA I

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Director

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Madrid, 2019

UNIVERSIDAD COMPLUTENSE MADRID

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ABBREVIATIONS

AB	Alamar Blue
AD	Autosomal dominant
AMP	Antimicrobial peptides and proteins
APCs	Antigen-presenting cells
AR	Autosomal recessive
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BKV	Polyomavirus BK
DC	Dendritic cell
Cfu	Colony forming units
CGD	Chronic granulomatous disease
CGH	Comparative genomic hybridization
CID	Combined immunodeficiency
CIITA	Class II Transactivator
CK	Cytokeratin
CMC	Chronic mucocutaneous candidiasis
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte antigen-4
CYBB	Cytochrome b beta chain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EBV-B cells	Epstein-Barr Virus-Transformed B cells

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
FOXA1	Forkhead box protein A1
GAF	Gamma-activated factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Gamma-activating sequence
GFP	Green fluorescent protein
GH	Growth hormone
GHIS	Growth hormone insensitivity syndrome
GOF	Gain-of-function
HIES	Hyper-IgE syndrome
HHV	Human herpes virus
HPV	Human papilloma virus
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes Simplex virus
hTERT	Human telomerase reverse transcriptase
ILCs	Innate lymphoid cells
IFN	Interferon
IFNαR	Interferon-α receptor
IFNγR	Interferon-γ receptor
Ig	Immunoglobulin
IKBKG	Inhibitor of kappa B kinase gamma
ICAM	Intercellular adhesion molecule
IL	Interleukin

iPSCs	Induced pluripotent stem cells
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-α/β sequence response elements
IU	International unit
JAK	Janus Associated Kinase
KD	Knock down
KO	Knock out
KSFM	Keratinocyte serum-free medium
LOF	Loss-of-function
MHC	Major histocompatibility complex
miRNAs	MicroRNAs
MOI	Multiplicity of infection
MSMD	Mendelian susceptibility to mycobacterial disease
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NEMO	NF-kappa-B essential modulator
NHU	Normal human urothelial cells
NGS	Next generation sequencing
NK	Natural Killer
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX2	NADPH oxidase
NTM	Nontuberculous mycobacteria
OAS	Oligoadenylate synthetase
OD	Optical density
PAMP	Pathogen-associated molecular pattern

PBMCs Peripheral blood mononuclear cells

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PFA Paraformaldehyde

PFU Plaque-forming unit

PHA Phytohemagglutinin

PID Primary immunodeficiency

PIV5 Parainfluenza virus 5

PIV5VΔC Attenuated recombinant strain of PIV5

PKR Protein kinase R

PMA Phorbol myristate acetate

PD-L1 / PD-1 Programmed death-ligand 1 / programmed death-ligand 1

Pfu Plaque forming units

PI Propidium Iodide

PMA Phorbol myristate acetate

Pol Polymerase

PPARγ Peroxisome proliferator activated receptor gamma

PR Partial recessive

PRRs Pattern recognition receptors

PRV-3 Parainfluenza virus type 3

P/S Penicillin-streptomycin

qPCR Quantitative real-time PCR

RNA Ribonucleic acid

RNS Reactive nitrogen species

RORγ Retinoic acid-related orphan receptor gamma

ROS Reactive oxygen species

RSV	Respiratory syncitial virus
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time-quantitative polymerase chain reaction
Sc	scramble control
SCID	Severe combined immunodeficiency
SE	Standard deviation
shRNA	Short hairpin RNA
SOCS	Suppressor of cytokine signalling
SPPL2A	Signal peptide peptidase-like 2A
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
TYK2	Tyrosine kinase 2
TZ	Troglitazone
UPK	Urothelium specific uroplakins
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
WT	Wild type
γc	Common gamma chain

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RESUMEN

Introducción

Las inmunodeficiencias primarias (IDP) son trastornos genéticos que ocasionan disfunción del sistema inmune y predisposición a infecciones. Las tecnologías de secuenciación de nueva generación han recientemente revolucionado el campo de las IDPs, identificando sus bases moleculares y orientando el diseño de nuevos tratamientos. De igual forma, el estudio de IDP monogénicas ha ayudado a descifrar el funcionamiento del sistema inmunológico, teniendo gran impacto en las áreas de autoinmunidad y cáncer.

Mediante el uso de secuenciación de nueva generación, nuestro grupo ha recientemente identificado una nueva IDP asociada a mutaciones hipomórficas con pérdida de función de la proteína de señalización Janus Associated Kinase 1 (JAK1). Las manifestaciones clínicas del paciente se caracterizaron por la presencia de infecciones recurrentes por micobacterias atípicas y carcinoma transicional de vejiga de alto grado, que ocasionó el fallecimiento del paciente durante la tercera década de vida.

JAK1 pertenece a una familia de tirosín quinasas esenciales para la transducción de señal a través de varios grupos de receptores de citoquinas, donde miembros de la familia JAK (JAK1, JAK2, JAK3 y TYK2) se asocian a receptores específicos con funciones no redundantes a nivel celular. La unión al receptor inicia la actividad quinasa de JAK que resulta en el reclutamiento y fosforilación de transductores de señal

y activadores de la transcripción (STAT), con la consiguiente inducción de expresión de genes específicos. Gracias al estudio de IDPs en modelos animales y en humanos se ha podido descifrar el rol de los diferentes miembros de la familia JAK.

La deficiencia de JAK1 no ha sido descrita previamente en humanos y los modelos en ratón han proporcionado poca información debido a la mortalidad perinatal por complicaciones neurológicas. Se conoce que JAK1 coopera con JAK3 en la transducción de receptores de citoquinas (IL-2, IL-4, IL-6, IL-7, IL-9, IL-15 e IL-21), y coopera con JAK2 y TYK2 en la señalización del IFNγR e IFNαR respectivamente, pudiendo desempeñar un importante rol en la defensa frente micobacterias y virus.

La susceptibilidad mendeliana a infecciones por micobacterias (MSMD) constituye un heterogéneo grupo de IDPs caracterizado por la predisposición a infecciones por micobacterias atípicas o de poca virulencia como la vacuna *Micobacterium bovis* Bacillus Calmette-Guerin (BCG). Se han descrito mutaciones en los genes *IFNGR1*, *IFNGR2, IL12B, IL12RB1, STAT1, ISG15, IRF8, IKBKG, CYBB, NEMO, TYK2, RORc/RORcT, SPPL2A* y *JAK1*, asociadas a MSMD. Se ha observado que dichas mutaciones afectan a la producción o la respuesta al IFNγ, directa o indirectamente, indicando que la integridad de la vía del IFNγ/IL-12 es importante para el control de la infección de microorganismos intracelulares como micobacterias. Sin embargo, aún no se ha podido identificar la etiología de un considerable número de pacientes.

El IFNα/β es esencial para el desarrollo de inmunidad anti-viral en humanos, a través de la vía de señalización JAK/STAT. En vista del rol de JAK1 en esta vía de señalización, resulta sorprendente que el paciente mostrara relativamente escasa susceptibilidad frente a virus. La susceptibilidad a infecciones virales ha sido previamente observada en defectos de la vía de señalización del IFNα/β, como son la deficiencia de IFNAR, STAT1, TYK2 y STAT2.

Hasta la fecha no se han descrito otros casos de carcinoma uroterial en pacientes con IDP asociada a defectos de la vía JAK1/STAT. Sin embargo se han reportado otros tipos de tumores en pacientes con defectos de la vía de señalización del IFNγ, incluyendo carcinoma cutáneo de células escamosas diseminado. Recientemente se ha asociado la presencia de mutaciones somáticas en el gen *JAK1* en algunos tipos de cáncer ginecológico y de vejiga, sugiriendo que defectos en la vía JAK1/STAT podría jugar algún rol en la patogénesis de cánceres epiteliales. En tal sentido, se postuló que defectos de la vía de señalización del IFNγ podría afectar funciones en las células uroteliales y en el desarrollo de mecanismos de evasión inmune tumoral.

El receptor de peroxisoma-proliferador-activado gamma (PPARγ) es un factor de transcripción implicado en mecanismos de diferenciación en células uroteliales. Se ha descrito que PPARγ opera mediante la inducción de transcripción de los factores de transcripción intermediarios FOXA1 e IRF1. IRF1 es miembro de la familia de factores de transcripción reguladora del IFN, pudiendo proporcionar un enlace entre la señalización de JAK1 y la función de las células uroteliales.

El propósito del proyecto consiste en determinar cómo la deficiencia de JAK1 conlleva el deterioro de la función de las células inmunes en humanos, abordando específicamente las funciones de JAK1 en la protección frente a micobacterias y virus, así como en la patogénesis del cáncer urotelial.

Materiales y métodos

A fin de investigar el rol de JAK1 durante la infección por micobacterias en células mieloides, se modeló la deficiencia de JAK1 en células THP1 mediante la utilización de vectores lentivirales que expresan secuencias de shRNA. Se analizó la capacidad de fibroblastos y células B inmortalizadas del paciente con deficiencia de JAK1, de desarrollar una respuesta anti-viral in vitro con cepas de virus parainfluenza 5 (PIV5), cepas atenuadas de PIV5 (PIV5VΔC) y virus de la estomatitis vesicular (VSV). A fin de investigar si la deficiencia de JAK1 podría promover el desarrollo de carcinoma urotelial mediante la afectación de diferentes mecanismos de evasión inmune tumoral. Se generó una línea celular urotelial hTERT con deficiencia de JAK1 mediante técnicas de shRNA. Además se investigó la expresión de factores de transcripción implicados en la diferenciación de células uroteliales humanas.

Resultados y discusión:

Se observó una reducción significativa en la fosforilación de STAT1 en las líneas celulares con deficiencia de JAK1 posterior a la estimulación con IFNγ, así como

menor inducción de la expresión de genes regulados por el IFNγ, demostrando disminución de la función de JAK1.

Mediante el uso de modelos de infección in vitro con BCG, se observó un incremento en la supervivencia micobacteriana posterior a la estimulación con IFNγ, en las células con deficiencia de JAK1 en comparación con el control, lo que se correlaciona con el fenotipo clínico del paciente. Los macrófagos activados por IFNγ son más resistentes a la infección por micobacterias mediante mecanismos de eliminación bacteriana como la inducción de apoptosis y maduración de fagosomas, los cuales se encontraron reducidos en la línea celular *knock down* (KD) posterior a la estimulación con IFNγ.

Se observó una respuesta anti-viral conservada posterior a la estimulación con IFNα tanto en fibroblastos del paciente con deficiencia de JAK1 como en el control. Estos resultados son consistentes con la ausencia de infecciones virales clínicamente graves en nuestro paciente, sugiriendo que la actividad residual de JAK1 podría ser suficiente para el control de la replicación viral. Sin embargo, células B inmortalizadas del paciente con deficiencia de JAK1 mostraron falta de protección posterior a la estimulación con IFNα, estableciendo un defecto en la respuesta al IFN tipo I, que varía entre diferentes tipos celulares.

En vista de que el paciente fue diagnosticado de carcinoma metastásico de vejiga a temprana edad, se postuló que la deficiencia de JAK1 podría predisponer al desarrollo

de carcinoma urotelial mediante la afectación de mecanismos de evasión inmune tumoral. Mediante la utilización de un modelo de células uroteliales con deficiencia de JAK1, se observó que la pérdida de la función de JAK1 afecta la inducción de apoptosis en respuesta al IFNγ. Las células KD también demostraron disminución en los niveles de expresión de la molécula de adhesión intracelular ICAM-1 en la superficie celular y, en menor medida, el complejo mayor de histocompatibilidad (MHC) I, posterior a la estimulación con IFNγ. Estos hallazgos se asociaron con resistencia a la lisis celular mediada por linfocitos, que es dependiente de la expresión de estas moléculas. Además se observó el requerimiento de JAK1 en la expresión de MHC II, involucrado en la presentación de antígenos tumorales. Estos hallazgos sugieren que las células uroteliales con deficiencia de JAK1 son más resistentes a la inducción de apoptosis mediada por IFNγ, al reconocimiento por parte de células del sistema inmune y muerte celular.

Las células KD demostraron disminución en los niveles de expresión del ligando 1 de muerte programada (PD-L1). Se ha reportado que mutaciones somáticas en la vía de señalización de IFNγ están asociadas con metástasis y una mayor resistencia a tratamientos bloqueantes anti-PD-L1/ PD-1 en varios tipos de tumores. Consistente con esto, nuestros hallazgos sugieren que mutaciones asociadas a la pérdida de la función de JAK1 constituyen un factor de riesgo para la menor expresión PD-L1 en células uroteliales tumorales, lo que podría interferir con la capacidad de respuesta al tratamiento con anti-PD-1.

Además de las funciones relacionadas con el sistema inmunitario, se observó un papel potencial de JAK1 en el proceso de diferenciación de células uroteliales. La estimulación con IFNγ mostró un efecto significativo en la inducción de los factores de transcripción IRF1 y FOXA1 en células uroteliales humanas. Dichos factores se han asociado al proceso de diferenciación de células uroteliales dependiente de la activación de PPARγ. En particular, IRF1 representa un mediador común para las vías de señalización de PPARγ e IFNγ, pudiendo afectar tanto en el fenotipo de diferenciación urotelial como las interacciones con el sistema inmunitario.

Conclusión

Estos hallazgos sugieren que la deficiencia parcial de JAK1 puede afectar diferentes vías de señalización, presentando un defecto dominante en la vía del IFNγ que resulta en susceptibilidad a infecciones por micobacterias. A pesar de evidenciar susceptibilidad frente a virus *in vitro*, ésta se presentó de forma variable dependiendo del tipo celular. Los hallazgos además resaltan funciones previamente desconocidas de JAK1 en el reconocimiento inmune y el proceso de diferenciación de las células uroteliales, proporcionando una plataforma para el desarrollo de nuevos biomarcadores y tratamientos dirigidos para el carcinoma urotelial.

SUMMARY

Primary immunodeficiencies (PIDs) are genetic disorders where components of the immune system are missing, predisposing to infection, autoimmunity and malignancy. Next generation genetic sequencing (NGS) has revolutionised the field of PIDs, identifying the molecular basis of inherited immune disorders. Using NGS, we recently reported the first description of hypomorphic loss of function mutations in human Janus Kinase 1 (*JAK1*) in a patient with recurrent atypical mycobacterial infections, early onset fatal high-grade urothelial carcinoma, and relatively minor viral infections.

JAK1 belongs to a family of widely expressed tyrosine kinases essential for signal transduction through different cytokine receptors. Individual family members (JAK1, JAK2, JAK3 and TYK2) have non-redundant roles in cell biology. Receptor binding initiates JAK kinase activity resulting in recruitment of signal transducers and activators of transcription (STAT) proteins and transcription of responsive genes. The roles of several members of the JAK family for immune cell function have been clarified through investigation of human deficiency states and murine models.

Selective susceptibility to weakly virulent mycobacteria, such as *Mycobacterium bovis* Bacillus Calmette-Guerin vaccine (BCG) or environmental mycobacteria species, is a genetically heterogeneous group of rare PIDs associated with mutations in the genes *IFNGR1, IFNGR2, IL12B, IL12RB1, STAT1, ISG15, IRF8, IKBKG, CYBB, NEMO, TYK2,RORc/RORcT, SPPL2A* and *JAK1* that have been shown to cause mendelian susceptibility to mycobacterial disease (MSMD). These mutations impair the production

of or the response to interferon-gamma (IFNγ), indicating that human IL-12/IFNγ mediated immunity, is essential to control intramacrophagic infections, such as mycobacteria. However, no clear genetic etiology has been identified for a number of patients.

IFNα/β is essential for the protective immunity to viruses in humans via JAK/STAT signalling. Susceptibility to viral infection has been observed in genetic defects of the IFNα/β signalling pathway (IFNAR, STAT1, TYK2 and STAT2). Given the reported role of JAK1 in signalling from IFNαR, it is surprising that our patient had relatively little susceptibility to viral infection.

To date, urothelial carcinoma has not been described as a feature of JAK-STAT pathway PIDs. However, other tumours have been reported in patients with defects in the IFNγ pathway, including cutaneous squamous cell carcinoma. Recently, somatic mutations in *JAK1* were seen in high-risk bladder cancer and gynaecological carcinomas, supporting the idea that defective JAK1 signalling could play a role in the pathogenesis of some epithelial cancers. We hypothesised that reduced IFNγ signalling may intrinsically impair urothelial cell functions and tumour immune evasionmechanisms. Peroxisome proliferator activated receptor gamma (PPARγ) is a transcription factor implicated in urothelial cell differentiation that operates by transcription of the intermediary transcription factors FOXA1 and IRF1. IRF1 is a member of the interferon regulatory transcription factor family that is responsive to

IFNγ stimulation and could provide a link between JAK1 signalling and urothelial cell function.

This project aimed to understand the disease mechanisms of JAK1 deficiency and specifically address the roles of JAK1 in host protection from intracellular bacterial, viral infections and the pathogenesis of bladder cancer.

Materials and methods

We investigated the role of JAK1 in myeloid cells during mycobacterial infection using a JAK1 deficient THP1 cell line generated using lentiviral vectors expressing short hairpin RNA (shRNA) sequences. We studied the ability of skin fibroblasts and EBV B cells from the patient with JAK1 deficiency to develop antiviral response in vitro, using parainfluenza virus 5 (PIV5), a highly attenuated recombinant strain of PIV5 (PIV5VΔC) and vesicular stomatitis virus (VSV). In order to investigate if JAK1 deficiency may promote urothelial carcinoma affecting different tumour immune evasion mechanisms, we generated a JAK1 deficient hTERT urothelial cell line using shRNA. We also investigated the expression of transcription factors involved in urothelial cell differentiation in normal human urothelial cells (NHU).

Results and discussion

JAK1 deficient cells exhibit reduced STAT1 phosphorylation following IFNγ stimulation and reduced induction of expression of interferon-regulated genes,

demonstrating loss of JAK1 function. Using in vitro infection models with BCG and salmonella, JAK1-deficient THP1 cells supported enhanced bacterial survival after IFNγ stimulation compared to control. IFNγ-activated macrophages were more resistant to mycobacterial infection by the induction of different mechanisms that promote mycobacterial killing, such as expression of IFNγ inducible genes (*IRF1* and *CIITA*), phagosome maturation and apoptosis, which were found to be reduced in the knock down (KD) cell line after IFNγ stimulation.

Given the reported role of JAK1 in signalling from IFNαR, we studied the ability of skin fibroblasts to develop antiviral response in vitro. We observed suppression of viral infection after IFNα stimulation in both patient and control fibroblasts. These results are consistent with the lack of clinically severe viral infections in our patient, suggesting that residual JAK1 activity may be sufficient to develop antiviral immunity. However, the patient's EBV-B cells showed lack of protection after stimulation with IFNα, establishing a failure of the type I IFN response that varies between cell types.

Considering that our patient presented metastatic bladder carcinoma at an early age, we postulated that JAK1 deficiency may also promote urothelial carcinoma affecting tumour immune evasion. Using a JAK1-deficient urothelial cell model, we observed that loss of JAK1 function impaired induction of apoptosis in response to IFNγ. KD cells also demonstrated reduced surface expression levels of ICAM-1 and to a lesser extent MHC class I following IFNγ stimulation, which was associated with resistance to lymphocyte-mediated cell lysis that is known to correlate with cell surface expression of these molecules. JAK1 was also required for expression of MHC class II that mediates

tumour and self-antigen presentation in non-professional antigen presenting cells. Together these data suggest that JAK-deficient urothelial cells are less susceptible to IFNγ-mediated apoptosis, immune cell recognition and immune-mediated cell death. It has been reported that damaging mutations in the IFNγ signalling pathway are associated with metastasis and higher resistance to the checkpoint blocking therapy with anti-programmed death-ligand 1 / programmed death-ligand 1 (PD-L1/PD-1) in a number of tumour types. Our data suggest loss of function JAK1 mutations are a risk factor for lower tumour cell PD-L1 expression in urothelial cells, which could impair responsiveness to anti-PD-1 therapy.

In addition to immune-related functions, we observed a potential role for JAK1 and IFNγ signalling in urothelial cell differentiation. We showed that IFNγ had a significant effect on the induction of the transcription factors IRF1 and FOXA1 in NHU, both known to be involved in urothelial cell differentiation induced by PPARγ activation. In particular, IRF1 is a common downstream mediator for PPARγ and IFNγ signalling pathways influencing both urothelial differentiated phenotype and immune cell interactions.

Conclusion

These findings suggest that the predominant effect of partial JAK1 deficiency is on the IFNγ pathway resulting in mycobacterial susceptibility. Although viral susceptibility was also observed in vitro, this varied according to cell type. The findings also highlight previously unknown roles for JAK1 in urothelial cell immune recognition and

differentiation, providing a platform for further development of novel biomarkers and targeted therapy for urothelial carcinoma.

INTRODUCTION

1. Primary immunodeficiency diseases

Primary immunodeficiencies (PID) are inherited disorders where components of the immune system are missing, predisposing to infection, autoimmunity and malignancy. Next generation sequencing (NGS) has allowed the identification of new monogenic forms of PID and around 320 different gene defects in humans has been described (1). Most of PIDs present with increased susceptibility to infection that could be restricted to a certain pathogen (e.g. *Candida sp, Mycobacterium sp*, etc) or extend to various pathogens. Nevertheless, with the increasing number of identified PIDs and better management of the infectious complications, many PIDs have been associated with additional clinical presentations such as autoimmunity and malignancy.

The classification of PIDs is constantly revised and updated by international societies' expert committees. In the last update, PIDs were classified into nine major categories on the basis of the affected immunological compartment: immunodeficiencies affecting cellular and humoral immunity, combined immunodeficiencies (CID) with syndromic features, antibody deficiencies, diseases of immune dysregulation, defects of the innate immunity, defects of phagocytes, auto-inflammatory disorders, defects of the complement system, among others (1).

We recently identified a new PID associated with compound heterozygous loss of function mutations in the signalling protein Janus-Associated Kinase 1 (JAK1) (2). This is the first reported case of human JAK1 deficiency. The clinical phenotype was

characterized by recurrent atypical mycobacterial disease and aggressive urothelial carcinoma that was fatal in the third decade of life. Recurrent atypical mycobacterial disease was the dominant immunodeficiency phenotype, grouping JAK1 deficiency with other diverse genetic defects of the IFNγ pathway as a cause of mendelian susceptibility to mycobacterial disease (MSMD).

In view of this, I specifically investigated the impact of partial JAK1-deficiency on interferon (IFN) signalling in immune cells in order to study the role of JAK1 on mycobacterial and viral susceptibility, and predisposition to bladder cancer. Subsequent sections will introduce the mechanisms and consequences of JAK/STAT signalling pathway in the immune system, with special emphasis on defects associated with MSMD, viral infection and malignancy.

2. Responses to cytokines and interferons that depend upon JAKs and signal transducers and activators of transcription (STAT) proteins

Cytokines have been found to play critical roles in cell growth and differentiation, metabolism, hematopoiesis, host defense, and immunoregulation. The type I and type II cytokine-receptor superfamily encompasses receptors that bind interferons, interleukins and colony-stimulating factors. These cytokines use the JAK/STAT pathway, which is recognized as an evolutionarily conserved signalling cascade involved in signalling and transcriptional regulation (3). The widely expressed JAK family of tyrosine kinases are essential for signal transduction through multiple cytokine receptors that signal through combinations of four JAKs (JAK1, JAK2, JAK3 and Tyrosine kinase 2 (TYK2)) and

seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6).

JAKs bind cytosolic domains of the cytokine receptors and signalling through the JAK/STAT pathway is initiated when a cytokine binds to its corresponding receptor. This leads to conformational changes in the cytoplasmic portion of the receptor initiating the activation of JAKs, which phosphorylate each other as well as the intracellular tail of the receptor subunits, creating docking sites that recruit downstream signalling molecules. This results in recruitment and phosphorylation of STATs and transcription of STAT-responsive genes (4). Upon activation, STATs bind to phosphorylated cytokine receptors and undergo tyrosine phosphorylation, allowing them to dimerize and translocate to the nucleus. This promotes nuclear accumulation of the STATs, DNA binding and activation of gene transcription. Once activated, STATs play a critical role in regulating innate and acquired host immune responses (4).

The JAK/STAT pathway regulates multiple cellular functions including growth, differentiation and homeostasis, although individual JAK/STAT molecules play specific roles in different cell types (3). JAK1 is involved in signalling by members of the IL-2 receptor family (IL-2R, IL-7R, IL-9R and IL-15R), the IL-4 receptor family (IL-4R, IL-13R), the gp130 receptor family (IL-6R, IL-11R, LIF-R, OSM-R CT-1R, CNTF-R, NNT-1R/BSF- 3R and Leptin-R) and class II cytokine receptors (type I IFN-R, type II IFN-R, IL-10R (4).

JAK1 is a ubiquitously expressed and is activated by a wide variety of cytokines, having impact beyond the immune system. The effect of complete JAK1 deficiency has been revealed by perinatal lethality in JAK1 knock out murine models, possibly as a result of neurological defects. The results from analyses of cells and tissues derived from JAK1 deficient mice demonstrated that JAK1 plays a critical role in mediation of biological responses to the major cytokine receptor subfamilies, and identified profound defects in the biological response to type I and type II IFNs in the context of complete JAK1 deficiency (5).

3. Lessons from JAK family knock out mice

The study of knock out (KO) mice has substantially advanced our understanding of the importance of different JAK family members in vivo. Findings in JAK family KO mice have demonstrated obligate relations between specific cytokine receptors and their JAK effectors although functional redundancy also exists. In specific circumstances, the absence of one JAK family kinase can be compensated for by another family member fulfilling the same signalling function, highlighting the complexity of the JAK-STAT pathway.

TYK2 and *JAK3* KO mice are viable and fertile, but display significant defects of immunity. Specifically, TYK2-deficient mice show prominently high sensitivity to infections and defective tumour surveillance. TYK2 cooperates with JAK1 and/or JAK2. In contrast with inactivation of other JAK family members, which completely

abrogates cytokine receptor signalling, loss of TYK2 reduces but preserves partial responses to cytokines including IL-12, IFNα/β and IFNγ (6,7)**.**

In contrast with the ubiquitous expression of TYK2, JAK3 expression is confined to haematopoietic tissues, myeloid and lymphoid cells and deletion of *JAK3* causes lymphopoietic defects that significantly impact lymphocyte development and manifest as severe combined immunodeficiency disease (SCID) in these mice (8,9). JAK3 cooperates with JAK1 for the signalling through the γc-cytokines (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21), which play distinct and non-redundant roles in the development and differentiation of T lymphocytes (10). While JAK3-deficiency in mice and humans causes comparable phenotypes, there is an interesting discrepancy between the phenotypes of the *TYK2* KO mice and humans lacking TYK2, suggesting differences interspecies. Cells derived from *TYK2* KO mice and *TYK2* deficient human exhibited defective IL-12, IL-23 and IFNα/β signalling, but additional severe defects in IL-6 and IL-10 signalling that were not observed in the mouse (11). These may indicate an evolutionary divergence in the receptor specificity or the capacity for compensatory signalling in different organisms (9).

Unlike TYK2 or JAK3, complete loss of JAK1 or JAK2 causes perinatal lethality attributed to neuronal defects in *JAK1* KO mice (5) and absence of definitive haematopoiesis in *JAK2* KO models (12). It is remarkable that like JAK1 and TYK2, JAK2 is ubiquitously expressed, but defects in JAK2 signalling are confined to HSC, erythroid and thrombopoietic signalling (9). Perhaps not surprisingly, neither complete germline JAK1 nor JAK2 deficiencies have been described in humans.

Although perinatal lethality severely limited examination of the signalling pathways mediated by JAK1 in vivo, some useful information has been determined. JAK1-deficient mice displayed reduced numbers of thymocytes, pre-B cells, and mature T and B lymphocytes but did not show alterations in other hematopoietic lineages. Embryonic fibroblasts derived from JAK1 null mice were unresponsive to IFNα or IFNγ, showing impaired major histocompatibility complex (MHC) class I expression and antiviral activity. Neither IFNα nor IFNγ protected *JAK1* null fibroblasts from viral infection with vesicular stomatitis virus (VSV). Macrophages from *JAK1* nulle embryos did not produce nitric oxide (NO) in response to either IFNα or IFNγ. The gp130 receptor family members also failed to provoke a biologic response in primary cardiomyocytes and neurons. No apparent structural heart abnormalities were found at the time of the perinatal death. In contrast, neurons from mice with JAK1 deficiency unable to respond to the ligands of the gp130 receptor family died by apoptosis, which could be the cause of the perinatal lethality observed (5). Together these findings suggest that other JAKs may be able to compensate to some degree for the absence of JAK1 in specific signalling cascades, but this compensation is insufficient to preserve full function in all cell types. Loss of JAK1 in conditional mice models has also shown that JAK1 is crucial for NK cell development and significantly affects NK cell-mediated tumour surveillance (13).

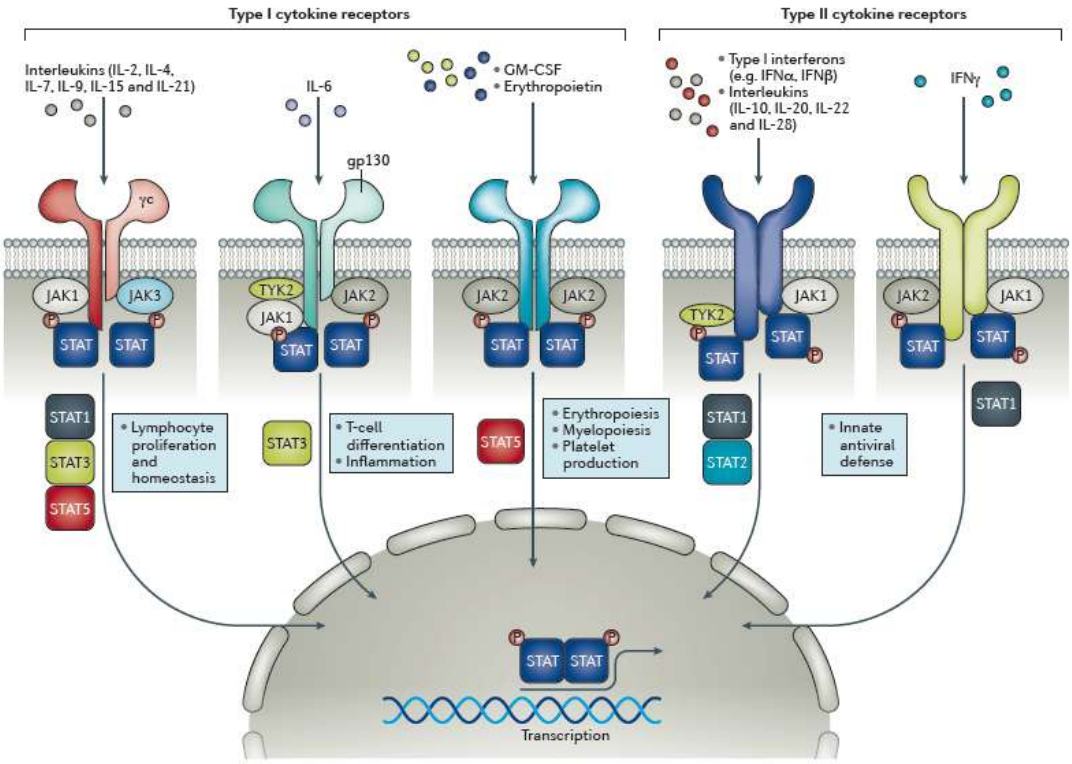


Figure 1. Type I and II cytokine receptors pathways (Adapted from Winthrop KL *et al*. Nat Rev Rheumatolo. 2017)

4. Consequences of JAK-STAT signaling in the immune system and human disease

The roles of several members of the JAK family for immune cell function have been clarified through investigation of human and murine deficiency states. Inherited variations in the JAK/STAT genes have been associated with increased risk of immune-mediated disease (14). To date, germline mutations in Janus kinases (JAK1, JAK3 and TYK2) and STATs (STAT1, STAT5B, STAT3) have been found in PID patients (15–19), providing answers to key questions about the function of these molecules.

In addition, somatic variations in JAKs and STATs have been associated with a variety of human diseases. JAKs have critical roles in hematologic cancers, mainly associated with the constitutive activation of JAKs and STATs (14). Somatic gain-of-function (GOF) mutations in the JAK2 kinase-like domain are associated with myeloproliferative diseases, including polycythemia vera, essential thrombocytopenia and myelofibrosis. Constitutive activation of JAK1, JAK2, JAK3, and TYK2, through GOF mutations or by constitutive cytokine production has been associated with a variety of haematological and solid organ malignancies (3,20–23).

4.1 Inborn Errors of Human JAKs and STATs

Loss-of-function mutations (LOF) of *JAK3* underlie severe combined immunodeficiency (SCID)

SCID is a genetically heterogenous group of conditions characterized by a lack of autologous T cells and extreme susceptibility to infections caused by a broad range of pathogens. The initial description of SCID was associated with null mutations in *IL2Rγ*, an X-linked SCID (X-SCID) (24). The IL-2RG or common gamma chain is a shared receptor subunit of the receptors for IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines direct the growth and maturation of natural killer (NK), T, and B cells. Their ability to mediate signal transduction upon ligand binding is dependent on the activation of JAK1 and JAK3 (25). Patients with either JAK3 deficiency or X-SCID fail to generate T and NK cells and have nonfunctional B cells. This highlights the importance of JAK3 signalling from common gamma chain (γc)-containing IL receptors for development of these lineages (15,16,26,27). Hematopoietic stem cell transplantation (HSCT) or gene

therapy offer a cure. However, a long-term risk of severe cutaneous human papilloma virus (HPV) infections persists, possibly related to persistent γc-deficiency in other cell types (28).

Whereas null mutations in *IL2Rγ* and *JAK3* are responsible for SCID, hypomorphic mutations in the same genes may cause other immunodeficiencies, ranging from life-threatening Omenn's syndrome to milder combined immunodeficiencies (29), which are even associated with prominent clinical features of immune dysregulation, including lymphoproliferation and autoimmunity (30,31).

Inborn errors of TYK2 immunity

TYK2 deficiency has been associated with increased susceptibility to mycobacterial and viral disease, as a result of impaired signalling from the IL-12/IL-23 and INFα/β receptors respectively. TYK2 deficiency was considered to be a genetic aetiology of hyper IgE syndrome (HIES), based on the observation that the first patient with inherited complete autosomal recessive (AR) TYK2 deficiency had atopy, susceptibility to cutaneous staphylococcal diseases and high serum concentrations of IgE, as seen in other genetic forms of HIES. However, another patient with TYK2 deficiency was susceptible to intramacrophagic bacteria, such as BCG and viral diseases, including recurrent cutaneous herpes simplex virus (HSV) and none features of HIES (17,18). These patients are immunologically and clinically similar to patients with a partial form of AR STAT1 deficiency, displaying impaired, but not abolished IFNγ and IFNα/β immunity and a particular susceptibility to diseases caused by intracellular bacterial and

viral pathogens. TYK2 is important for cellular responses to IL-12 and IFN-α/β, providing a plausible basis for the susceptibility to mycobacteria and viral infections (31).

It has been recently described that homozygosity for the *TYK2* P1104A variant in humans impaired cellular responses to IL-23 affecting IFNγ production, making these patients prone to tuberculosis infection and identifying *TYK2* P1104A as a more common cause of MSMD. However the response to IFNα was only slightly reduced and the response to IL-12 was normal. These findings suggest that hypomorphic mutations can have different impact on JAK partner activation and also depend on the cytokine receptor and STAT involved. They also highlight the complexity of the JAK-STAT signalling, where redundancy and residual activity could induce a normal gene expression pattern, depending on the affected pathway and cell type (32).

JAK1 GOF causes immune dysregulatory and hypereosinophilic syndrome

The p.A634D *JAK1* GOF mutation was reported to cause a systemic immune dysregulatory condition, including skin inflammation, hepatosplenomegaly, autoimmunity and eosinophilia with eosinophilic infiltration of the liver and gastrointestinal tract, associated to activation of the JAK/STAT pathway. This GOF mutation was previously identified as a somatic mutation in malignant conditions (19). At baseline and following stimulation, patient cells showed high levels of downstream pSTAT1 activity through STAT1 phosphorylation; and treatment with ruxolitinib significantly decreased this responsiveness. After one month of treatment with

ruxolitinib, patients showed clinical resolution of the dermatitis and hepatosplenomegaly, and had significantly reduced eosinophilia.

Inborn errors of STAT1 immunity

STAT1 is a transcription factor involved in cellular responses mediated by cytokines including IFNs. Human STAT1 is a key molecule required for cellular responses to IFNα/β and IFNγ pathways (33). Different forms of inherited STAT1 deficiency have been described in humans: bi-allelic mutations cause AR complete or partial STAT1 deficiency; mono-allelic mutations cause autosomal dominant (AD) STAT1 deficiency or AD STAT1 gain of activity (34,35).

AR complete STAT1 deficiency is characterized by the absence of protein expression and abolished cellular responses to antimycobacterial IFNγ and antiviral IFNα/β and IFNλ. The patients' cells did not respond to IFNγ and IFNα in terms of GAF and interferon-stimulated gene factor 3 (ISGF3) activity. The cells were unable to control the replication of the viruses tested in vitro, following treatment with IFNα. Patients with AR complete STAT1 deficiency have a life-threatening susceptibility to both mycobacteria and viruses (36–38). Partial recessive (PR) STAT1 deficiency is conferred by hypomorphic mutations of *STAT1*. The response to IFNγ and IFNα is impaired but not abolished, and patients are susceptible to both intracellular bacteria and viruses (39,40). AD LOF mutations of *STAT1* were shown to be associated with the impairment of IFNγ responses and MSMD (41,42). This partial STAT1 deficiency affects the IFNγ signaling pathway, which resulted in susceptibility to mycobacterial infection but

normal viral control. Whereas AR complete STAT1 deficiency blocks both IFNγ and IFNα signaling, resulting in susceptibility to viral and mycobacterial infections, patients with partial STAT1 deficiency are broadly susceptible to viruses, including HSV infections (37,43). Unlike MSMD patients with *STAT1* mutations, whose outcome is favorable, patients with complete STAT1 deficiency died in the absence of HSCT.

On the other hand, heterozygous GOF mutations in *STAT1* have been associated to fungal diseases, mainly chronic mucocutaneous candidiasis (CMC) (44,45). These mutations are GOF, in terms of phosphorylation and gamma-activating sequence (GAS)-binding activity; the cells of patients display a stronger response to IFNγ, IFNα and IL-27 (46). Excessive STAT1 activation causes exaggerated responses to IFNγ and antagonize STAT3-mediated induction of IL-17, inhibiting the development of IL-17-producing T cells, that are important for host defence against *Staphylococcus aureus* and fungal infections (47). These patients are also susceptible to other infections, autoimmunity and malignancy (14). *STAT1* is an example of a gene for which LOF mutations have been shown to cause certain infectious diseases, whereas GOF mutations cause other infectious diseases (31).

Inborn errors of STAT3

Germline heterozygous LOF mutations in *STAT3* cause HIES that is characterized by eczema, staphylococcal infections, pneumonias, CMC and high levels of IgE, along with nonimmunologic features (48,49). STAT3 was initially recognized as a signal transducer for IL-6 and epidermal growth factor (EGF). STAT3 is directly involved in

signalling from a multitude of haematological and extrahaematological receptors, especially those using the common β-chain, gp130 (50). Other classes of receptors are known to activate STAT3, which has been implicated in the signal transduction pathways involving γc-dependent cytokines, type I and II IFNs, the IL-10 family of cytokines, IL-12 and IL-23, receptor tyrosine kinases, and other stimuli, depending on the cell type (51). STAT3 is also required for the development of T-helper (Th) 17 cells (IL-17 CD4 T cells) and thus is profoundly impaired in patients with HIES (52).

Germline LOF mutations in *STAT3* cause immunodeficiency, whereas somatic GOF mutations in *STAT3* are associated with large granular lymphocytic leukemic, myelodysplastic syndrome, and aplastic anemia (31). Recently, germline heterozygous GOF activating mutations in *STAT3* have been described to cause early-onset autoimmune syndromes and autoimmune lymphoproliferative disease. Patients exhibited a variety of clinical features, with most having lymphadenopathy, autoimmune cytopenias, multiorgan autoimmunity (lung, gastrointestinal, hepatic, and endocrine dysfunction), infections, and short stature. Functional analyses demonstrate that these GOF mutations lead to secondary defects in STAT5 and STAT1 phosphorylation and the regulatory T-cell compartment. This is consistent with reports showing that STAT3 can antagonize some of the functions of STAT5 related to regulatory T (Treg) cell differentiation. Treatment targeting a cytokine pathway that signals through STAT3 led to clinical improvement in some patients (53,54).

Inborn errors of STAT5B, immunodeficiency and immune dysregulation

Patients with STAT5B homozygous mutations have been reported in association to short stature and growth hormone (GH) insensitivity syndrome (GHIS), dysmorphism, severe susceptibility to various pathogens including opportunistic infections, autoimmune manifestations and eczema. These patients have variable lymphocyte counts and normal levels of immunoglobulins (55).The STAT5B gene encodes a key component of the IL-2R signalling pathway and has non-redundant functions in growth and immunity in humans. The association of immunodeficiency and autoimmunity in patients with STAT5B homozygous mutations reflects the biological role of IL-2-mediated signalling (56).

5. Innate and adaptive immunity

The human immune system can be divided into two broad components: the innate immune system and the adaptive immune system, which work in tandem to provide resistance to infection. Innate immunity refers to non-specific protective mechanisms that act as the first line of defense against pathogens. All nucleated cells have anti-infective mechanisms that can be amplified by cytokines such as IFNs.

Innate immunity includes neutrophils, macrophages, dendritic cells (DC), NK cells, and NK T cells in conjunction with natural barriers (mostly skin and gastrointestinal and respiratory mucosa), as well as antimicrobial agents, opsonins and cytokines. Innate immune cells develop immune responses through the recognition of diverse pathogens by different pattern-recognition receptors.

Adaptive immunity implies a highly specific immune response to infection, but it requires up to several days to generate a response. Adaptive immunity exhibits antigenic specificity against the pathogen and has the ability to develop memory against previously encountered pathogens, responding more rapidly upon a second exposure. The two major cell types that that participate in adaptive immunity are the T and B lymphocytes, T cells play a central role in cell-mediated immunity and B cells mediate humoral immunity (57).

Cytokines acting through the JAK-STAT pathways are critical for the development and function of diverse lymphoid cell subsets. In particular, different members of the common gamma chain cytokine receptor family that utilise JAK1 and JAK3 are important for development, homeostasis and function of T, B and innate lymphoid cells. The common γ-chain (γc), involved in the signalling of the cytokines IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, plays essential roles in T cell development and differentiation. Both CD4 helper T-cells and CD8 cytotoxic T-cells depend on JAK3/JAK1 signalling for development and loss of JAK3 leads to SCID (58). IL-7 is essential for T cell development and survival, but other γc cytokines also play critical roles in T cell development at later stages. IL-2 signalling is important for Foxp3+ regulatory T cell development (59) and IL-4 signalling plays a critical role in the development of innate CD8 T cells in the thymus (60). IL-15 signalling is necessary for memory T cell differentiation (61) and IL-21 signalling promotes development of effector CD4 T cells, such as follicular helper T cells and IL-17-producing Th17 cells (10,62).

During their development, B-cells undergo specification, followed by expansion and selection. These processes are mediated by regulated gene expression programmes and rearrangements of immunoglobulin (Ig) genes. Many of these processes are initiated by cytokines, chemokines, and cell–cell contacts, involving the PI3K, MAPK or JAK-STAT signalling pathway. Il-7 is essential for commitment to the B-cell lineage and for orchestrating the Ig recombination machinery (63). This suggests that although B-cell development proceeds in patients lacking specific components of the IL-7R pathway (common γc, JAK3 or IL7Ra), function is impaired.

Innate lymphoid cells (ILCs) are a group of lymphocytes that lack antigen specific T or B-cell receptors. ILC have the ability to regulate and amplify the immune response through a variety of effector functions (64,65) and they can be divided into three groups based on their pattern of cytokine expression (66). NK cells and other IFNγ-producing fall in the group type 1, type 2 consist of cells producing IL-13 and IL-5, and the type 3 group comprises cells producing IL-22 and/or IL-17. Generation and development of ILC functional diversity depends on a complex network that controls both ILC and Th cell differentiation (67). A common feature of ILCs is the requirement for IL-15 and IL-7 (65). The non-redundant function of these two cytokines makes the JAK-STAT pathway the main signalling cascade involved in ILC development. Supporting this, no ILCs have been found in patients with JAK3 deficiency, highlighting the importance of the JAK-STAT pathway on the development of multiple lymphocyte subsets (58).

5.1 Interferons

IFNs were the first cytokines discovered, characterized and used therapeutically (68,69). IFNs play a major role in promoting the transition from innate to adaptive immune responses and have profound immunomodulatory activity (70). IFNs drive the upregulation of class I MHC molecules and components of the antigen-presenting machinery. IFNs also help to activate NK cells by complex processes including the upregulation of perforin and granzymes (71). IFNs are often profoundly cytostatic, inducing a growth arrest and apoptosis in target cells (72,73); and this helps to eliminate infected cells (74).

There are three types of IFNs: Type I, II and III. Type I IFN includes IFN-α, IFNβ and others (IFNε, IFNκ, IFNω, IFNδ, and IFNτ) of unknown significance in humans. Most nucleated cells can produce IFN α/β and respond to it through the ubiquitously expressed type I IFN receptor. IFNγ is the only member of type II IFNs. IFNγ is predominantly synthesized by T cells and NK cells in response to the recognition of infected cells, and can act on broad ranges of cells that express the IFNγ receptor. Type III comprises IFNλ and this IFN signal through a receptor complex consisting of IL-10Rβ and IL-28Rα chains. Type III IFN is directly induced by virus infection and triggers similar antiviral effects to type I IFN (75).

Table 1. Comparison of human type I, type II and type III IFNs

Properties	Type I IFN (IFNα/β)	Type II IFN (IFNγ)	Type III IFN (IFNλ)
Members	IFNα, IFNβ, IFNε, IFNκ, IFNω	IFNγ	IFNλ1/2/3/4
Producing cells	All nucleated cells	T,B,NK,NKT cells and APCs	All nucleated cells
Responding cells	All nucleated cells	All nucleated cells	Lung, intestine and liver epithelial cells
Stimuli	DAMPs and PAMPs	IL-12/15/18, type IFN and PAMPs	DAMPs and PAMPs
Signalling molecules	TYK2, JAK1, all STATs	JAK1, JAK2, STAT1, STAT3	TYK2, JAK1, STAT1, STAT2
Transcription factor binding site	ISRE (canonical) GAS (non-canonical)	GAS (canonical) ISRE (non-canonical)	ISRE
Functions	Antiviral, antiproliferative, regulation of cell survival and immunoregulation	Antiviral, antiproliferative, immunomodulatory and antitumour response	Antiviral response, mucosal immunity

Adapted from Castro *et al*. Front Immunol. 2018

5.1.1 IFNγ signalling

IFNγ is one of the most important endogenous mediators of immunity and inflammation. IFNγ plays a key role in macrophage activation, inflammation and host defense against intracellular pathogens, Th1 responses and tumor surveillance. IFNγ orchestrates leukocyte attraction and directs growth, maturation and differentiation of many cell types, in addition to enhancing NK cell activity and regulating B cell functions such as immunoglobulin (Ig) production (76–81).

IFNγ signals mainly through the JAK/STAT pathway to achieve transcriptional activation of IFNγ-inducible genes. STAT1 is the major STAT protein activated by IFNγ. Many IFNγ functions are mediated by direct activation of immune effector genes by STAT1, including genes encoding anti-viral proteins, microbicidal molecules, phagocytic receptors, chemokines, cytokines, and antigen presenting molecules (82,83).

IFNγ signals through the IFNγ receptor, a heterodimer encoded by genes *IFNGR1* and *IFNGR2*. This membrane-bound receptor is composed of 2 chains (IFNγR1 and IFNγR2) which are constitutively bound to their respective JAKs, JAK1 and JAK2. The intracellular region of IFNγR1 includes a JAK1 constitutively binding site and a STAT1 docking site when it is phosphorylated. The intracellular region of IFNγR2 contains a JAK2 binding site, which associates with JAK2 constitutively (84). After IFNγ binding, two IFNγR1 subunits form a homodimer and the two IFNγR2 subunits are recruited to the IFNγR1 dimer. The binding of IFNγR2 to IFNγR1 brings the two Janus tyrosine kinases into physical proximity and initiates the tyrosine kinase activity, which phosphorylates and activates the STAT1 docking site, resulting in the activation of JAK1 and JAK2. Upon activation, JAKs trans-phosphorylate each other at tyrosines within the kinase domain and phosphorylate the cytoplasmic tail of the receptor. This allows recruitment of STAT1, which exists in a latent state in the cytoplasm. The phosphorylated STAT1 dissociates from the IFNγ receptor complex. The SH2 domain of each STAT1 binds with the phosphorylated tail of the other STAT1 (83,85), and the active STAT1 homodimer translocates to the nucleus leading to a complex that can bind nuclear DNA and regulate the transcription of IFNγ-induced genes. The STAT1 homodimer binds GAS in the genome (86,87), driving gene expression.

One of the major primary response genes induced by STAT1 signalling is the transcription factor interferon-regulatory factor 1 (IRF1), a member of the IFN regulatory transcription factor family (88). IRF1 leads the transcription of a large number of secondary response genes (89). The JAK/STAT signalling pathway is regulated at several levels by positive and negative mechanisms. In particular, deregulation or inhibition of the JAK/STAT pathway leads to lowered immunity and is often associated with increased tumorigenesis or metastatic dissemination (75,90,91). Transcriptional activity of STAT1 is augmented by MAPK-mediated phosphorylation of a serine residue in the carboxy-terminal transcription activation domain, and the amplitude of activation is fine tuned by feedback inhibition mediated by various negative regulators of JAK/STAT signaling such as the suppressor of cytokine signalling (SOCS) 1 (82).

5.1.2 IFN-α/β signalling

Most nucleated cells can produce IFN α/β and respond to it through the ubiquitously expressed type I IFN receptor. Type I IFNs are secreted by infected cells and constitute the major component of the innate immune system protecting against viral infection. IFN α/β production is induced after the sensing of microbial products by pattern recognition receptors (PRRs) and by cytokines. They induce cell-intrinsic antimicrobial states that limit the spread of infectious pathogens, particularly viruses. They modulate innate immune responses to promote antigen presentation and NK cell functions, and activate the adaptive immune system promoting the development of high-affinity antigen-specific T and B cell responses (74,92).

IFNα/β binds a heterodimeric transmembrane receptor, the IFNα receptor (IFNAR), which is composed of IFNαR1 and IFNαR2 subunits. IFNαR engagement was shown to activate the receptor-associated JAK1 and TYK2, which phosphorylate the latent cytoplasmic STAT1 and STAT2 (93,94). The phosphorylated STAT1 and STAT2 dimerize and translocate to the nucleus, where they assemble with IFN-regulatory factor 9 (IRF9) to form a trimolecular complex called IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to the IFN-stimulated response elements (ISREs), and activate the transcription of ISGs. ISG-encoded proteins restrain pathogens by several mechanisms, including the inhibition of viral transcription, translation and replication, the degradation of viral nucleic acids and the alteration of cellular lipid metabolism (92).

The canonical type I IFN signalling pathway components IFNAR, JAK1, TYK2, STAT1, STAT2 and IRF9 are widely expressed and thus most cell types are competent to mount type I IFN-dependent responses. Immune cells can respond rapidly to low levels of type I IFNs, a capacity that is maintained under homeostatic conditions in which small amounts of IFNβ maintain high basal expression levels of STAT1 and IRF9 that rapidly mobilize effective antimicrobial programmes (92). The magnitude of basal IFNαR signalling is restrained by opposing mechanisms that limit expression of IFNαR-JAK-STAT signalling components. These suppressive mechanisms include the pausing of RNA polymerase II (Pol II) at genes that encode IFN pathway components and the induction of microRNAs (miRNAs) that destabilize or suppress translation of the corresponding transcripts (95,96). It has been shown that the relative expression of STATs is an important determinant of the pattern of IFNαR-induced STAT activation, for example STAT3 restrains STAT1-mediated inflammatory signalling downstream of

IFNAαR. Shifts in the balance of activation of distinct STAT complexes downstream of IFNαR mediates its context-dependent function by altering the balance between antiviral, pro-inflammatory, suppressive and anti-proliferative functions (92).

6. Protective immunity to mycobacterial infection

Tuberculosis infects close to 2 billion people globally and causes about 10 million active cases with about 3 million deaths per year (97). In contrast, the nontuberculous mycobacteria (NTM), organisms of low intrinsic virulence, cause an unrecognized number of infections and the majority are controlled by the host immune system. Severe infections caused by the NTM are a signal of significant immune defects (98). The integrity of the IFNγ circuit is necessary for an effective immune response to intra-macrophagic pathogens, especially Mycobacteria. Some patients display a selective susceptibility to poorly virulent mycobacteria such as BCG vaccines and environmental NTM, and more rarely by other intra-macrophagic pathogens such as *Salmonella sp* (99–101).

The innate immune system of the host and the virulence of mycobacteria are two key components that determine the outcome of the mycobacterial infection. Facultative intracellular bacteria can survive and replicate inside host cells, most frequently macrophages. This group includes *Salmonella, Shigella, Listeria monocytogenes, Francisella tularensis* and *Mycobacterium sp*. (102). Depending on host and bacterial factors, the mycobacteria will multiply and destroy the infected macrophages and infect more cells, or be eliminated by the macrophage phagosomes. The host invasion by

replicating pathogens demands a rapid response provided by components of the innate immune system that consists of non-phagocytic cells, phagocytic cells in the circulation and tissues, complement and plasma proteins. These components play different complementary roles in host defense against intracellular microbes (102).

Microbes are first recognized by PRRs at the plasma membrane of immune cells, followed by phagocytosis and the gradual maturation of the phagosome into a phagolysosome, where microbes are digested. PRRs recognize and facilitate internalization of microbes through phagocytosis in macrophages, DCs, and neutrophilic granulocytes. Receptor-mediated opsonic or non-opsonic phagocytosis may occur depending on whether or not the microbe is coated by soluble PRRs like complement and antibodies (103). The recognition of mycobacterial components by innate immune cells through different PPRs induces a cytokine response that can promote early control of the infection (104). Subsequently, infected macrophages migrate to the lymph nodes, present bacterial antigens through the MHC class II molecules to naïve CD4+ T-lymphocytes, and activate Th1 adaptive cellular immunity (83).

IFNγ is essential for the activation of phagocytic cells to kill mycobacteria. IFNγ activates innate responses by augmenting inflammatory cytokine and chemokine production, microbial killing and antigen presentation by mononuclear phagocytes such as macrophages. Immune cell activation by IFNγ is dependent on its activation of the transcription factor STAT1, which activates transcription of ISGs that play a key role in IFNγ-mediated functions (105). IFNγ is the most important cytokine during the early

phase of infection with intracellular pathogens; it acts synergistically with bacterial products to activate various effector bactericidal or bacteriostatic mechanisms in infected phagocytic and non-phagocytic cells. The induction of a functional Th1 response is dependent on IL-12, which is produced by antigen-presenting cells (APCs) after exposure to the pathogen at the initiation of the immune response (102). IFNγ can be produced by CD4 and CD8 T cells, NK cells and infected macrophages, creating a positive loop between T cells and APC, which enhances the microbicidal capacity (106–108).

6.1 Mycobacterial killing mechanisms

The macrophage is the site of elimination and bacillus replication, and different immune intracellular killing mechanisms mediate the elimination of intracellular pathogens. The main mycobacterial killing mechanisms are phagocytosis, production of cytokines, reactive oxygen and nitrogen species, phagosome maturation and cell death. Intracellular bacteria such as *Mycobacterium* and *Salmonella typhi* can survive inside mononuclear phagocytes (109), and within cells, bacteria are protected from humoral attack mechanisms.

Phagosome maturation

Following phagocytosis by host cells, mycobacteria are localized in membrane-bound vesicles, the phagosomes. The newly formed phagosome proceeds through numerous steps of maturation. Phagosomes mature into late phagosomes, and then into phagolysosomes via sequential fusion with pre-existing lysosomes (102). Antimicrobial

mechanisms of the mature macrophage phagosome include acidification, production of AMPs, activation of the inducible nitric oxide synthase 2 (NOS2) and NADPH oxidase (NOX2), and degradative enzymes, such as cathepsins. Low pH, oxidative stress, and nutrient deficiency in the maturing phagosome act as antimicrobial pathways (103,110).

Within the lysosomal vacuoles are potent hydrolytic enzymes that function optimally at acidic pH (4.5-5.0) and are capable of degrading microorganisms. The lysosomal acidic environment is maintained by a membrane adenosine triphosphate (ATP)-dependent proton pump that is recruited to phagosomes to facilitate luminal acidification and to activate lysosomal hydrolases and cathepsins that degrade phagolysosomal content. The degradation of intracellular microorganisms by intralysosomal acidic hydrolases constitutes a significant antimicrobial mechanism of phagocytes. In addition, the process of microbial degradation by lysosomes results in the generation of antigenic peptides suitable for presentation by class II MHC molecules and activation of CD4+ T lymphocytes (102).

Reactive oxygen and nitrogen species (ROS and RNS) production

Phagocytosis of microbes activates NOS2 and NADPH oxidase that results in the production of RNS/ROS respectively (111). In the phagosome, NO and ROS can spontaneously react to generate highly reactive intermediates that destroy microbial membrane lipids, DNA, and thiol- and tyrosine residues by oxidation. In humans, polymorphisms in the NOS2 or CYBB (coding for gp91phox) genes have been associated with an increased susceptibility to TB (103,112). The role of NO as a

primary antimicrobial effector molecule in macrophages and non-phagocytic cells has been established in vitro in murine models. However, much less is known about the role of NO in protection of humans against intracellular bacteria (102). Recently it has been shown that IFNγ induced apoptosis in mycobacteria-infected macrophages could be NO dependent and results in the killing of intracellular mycobacteria (113).

Apoptosis

Macrophage apoptosis represents an important innate defense mechanism against intracellular mycobacterial infection. Previous publications have shown that IFNγ is involved in apoptosis of immune cells during infection with mycobacteria (114,115). Apoptosis is programmed cell death characterized by cytoplasmic shrinking, cell rounding, chromatin condensation, DNA fragmentation and membrane blebbing. Apoptosis can be initiated by cell extrinsic pathways (which are mediated by death receptors) or cell intrinsic (mitochondrial) pathways, both of which culminate in the activation of the effector caspases. Apoptosis can also be initiated by cytotoxic T lymphocytes (CTLs) or NK cells that deliver granzymes, which activate apoptotic caspases. Apoptosis has been considered intrinsically bactericidal. IFNγ induced NO-mediated apoptosis has been described as a defense mechanism of activated macrophages against *M. tuberculosis* (116).

Mendelian susceptibility to mycobacterial disease (MSMD) mutations in the IFNγ signalling genes

MSMD is included in the PID classification by the IUIS (International Union of Immunology Societies) in the VIth group of defects in intrinsic and innate immunity (117). There are different genetic aetiologies of MSMD, mainly associated to defects in genes encoding proteins involved in IFNγ immunity. These mutations impair the production of or the response to IFNγ, either directly or indirectly, indicating that the IFNγ pathway is critical for the confinement of mycobacterial infection in humans (31). Patients with defects of the IFNγ pathway may display a selective susceptibility to poorly virulent mycobacteria such as BCG vaccine and environmental NTM, and other intra-macrophagic pathogens (101,106,118). Nevertheless, genetic aetiology in approximately half of patients with MSMD remains unknown (119).

There is high genetic heterogeneity and to date mutations in different genes (*IFNGR1, IFNGR2, IL12B, IL12RB1, STAT1, ISG15, IRF8, IKBKG, CYBB, NEMO, TYK2, RORc/RORcT, SPPL2A* and *JAK1*) have been shown to cause MSMD (106,120,121). There are different types of MSMD depending on which gene is affected, the impact of the mutation (null or hypomorphic), the mode of transmission in the family (dominant or recessive), the expression of the mutant allele (absent or detectable), or the function affected by the mutation (one domain or another, in the case of a detectable protein). The most severe forms of MSMD lead to early-onset, disseminated, life threatening mycobacterial disease, with an outcome that leads to death if HSCT is not performed, whereas the mild forms can have a late onset or even remain clinically silent because of incomplete penetrance and clinical manifestations are highly variable (63,65).

Mycobacterial infections are the most common infections in patients with MSMD. However, the clinical phenotype extends to syndromic MSMD, including other infections associated with a more complex cellular phenotype. Some examples such as AR STAT1 and TYK2 deficiencies include infections caused by other intramacrophagic bacteria, fungi and parasites. Viral infections have also been reported (cytomegalovirus (CMV), human herpes virus 8 (HHV8), parainfluenza virus type 3 (PRV-3), respiratory syncitial virus (RSV) and varicella zoster virus (VZV)). Some cases of malignancies, namely B-cell lymphoma, esophageal carcinoma, cutaneous squamous cell carcinoma, Kaposi sarcoma, liver cancer and pineal germinoma have also been reported (Table 2) (101,122–126).

Table 2. Clinical diseases allelic with MSMD at the STAT1 IFNγR1, IFNγR2 and TYK2 loci

Disease	Mycobacterial	Viral	Other pathoges	Malignancy
STAT1 LOF AR -Complete -Partial	+++ ++	++ +	+	
STAT1 LOF AD	++			Liver carcinoma
IFNγR1 LOF AR -Complete -Partial	+++ +	++ +	+	B cell lymphoma Pineal germinoma
IFNγR1 LOF AD	++			
IFNγR2 LOF AR -Complete -Partial	+++ +			Cutaneous squamous carcinoma
IFNγR2 LOF AD	+			
TYK2 LOF AR	++	+	+	

Adapted from Bustamante *et al*. Semin Immunol. 2014.

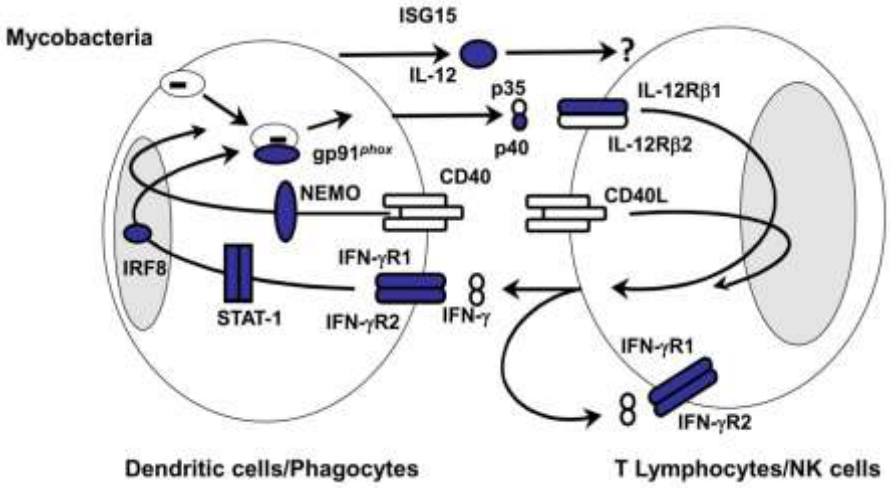


Figure 2. Cells producing and responding to IFNγ

Proteins for which mutations in the corresponding genes have been identified and associated with Mendelian susceptibility to mycobacterial diseases (MSMD) are indicated in blue. MSMD-causing mutations of *IFNGR1*, *IFNGR2*, *STAT1*, *IRF8* and *CYBB* impair the action of IFNγ. MSMD-causing mutations of *IL12B*, *IL12RB1*, *ISG15*, *IRF8* and *NEMO* impair the production of IFNγ (Adapted from Bustamante J. *et al*. Semin Immunol. 2014).

7. Anti-viral immunity

IFNα/β upregulates the expression of several genes inducing an antiviral state. A subset of genes is required to limit viral replication, these genes encode enzymes dsRNA-dependent protein kinase R (PKR), oligoadenylate synthetase (OAS) and Mx, which show antiviral activity (74,127,128). PKR and OAS are enzymes whose activities are dependent upon viral co-factors such as dsRNA, when the co-factors are provided; the enzymes can produce changes in cellular function through translational arrest. Mx encodes GTPases with antiviral activity against a wide range of RNA viruses, preventing the transport of the viral nucleocapsids into the nucleus and viral replication (129). Other IFN-inducible factors promote the presentation of viral antigens to the adaptive immune response by upregulating MHC class I and the antigen-processing

machinery. IFNα/β also has immunomodulatory functions, by promoting the maturation of DCs, upregulating the activities of NK cells and CD8+ T cells (74).

There are different ways by which viruses evade the IFN response: interfering globally with host cell gene expression and/or protein synthesis, minimizing IFN induction by limiting the production of viral pathogen-associated molecular patterns (PAMPs) and/or by specifically blocking IFN-induction cascades, inhibiting IFN signalling blocking the action of IFN-induced enzymes with antiviral activity; and having a replication strategy that is insensitive to the action of IFN.

7.1 PIDs associated to susceptibility with viral diseases

Some immunodeficiencies enhance susceptibility to disease with a specific virus or family of viruses, whereas others predispose to diseases with viruses and other microbes. Susceptibility to severe viral diseases is encountered in PID with deficient innate and/ or adaptive immune responses (130). Theses PIDs can be divided into two categories: disorders caused by defects in the adaptive immune system (lymphocyte-derived cellular and antibody responses) and those caused by defects in the innate immune system (Toll-like receptors (TLRs), IFNs and NK cells).

7.1.1 PIDs resulting from defects in the IFN pathway

PIDs involving the IFN pathway include those that affect the production of IFN and those that affect the response of cells to IFN (mutations associated to defects in the IFN

receptor and signalling molecules). These immunodeficiencies predispose individuals to infection with intracellular pathogens, such as viruses, mycobacteria, and salmonella (131). X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency results from mutations in *NEMO*. Defects in this protein predispose individuals to infections with viruses and bacteria. The viral infections in these patients have been caused primarily by herpesviruses (CMV, HSV) (132). *STAT1* mutations result in failure of type I IFNs to induce expression of IFN inducible genes. Patients with AR complete STAT1 deficiency had disseminated BCG disease and fatal disseminated viral infections, as a consequence of impaired IFNγ and IFNα/β signalling that fails to inhibit virus replication. Patients with partial AR STAT1 deficiency also had severe CMV and VZV infections (37,133). TYK2 deficiency is associated with recurrent cutaneous viral infections, BCG lymphadenitis, and disseminated salmonella bacteraemia, due to defects in IL-12 and IFNα/β signalling (17). Patients with STAT2 deficiency usually present a mild phenotype with susceptibility to some viral infections, predominantly disseminated vaccine-strain measles. Mutations within *STAT2* drive a profound defective innate IFN response. However, the viral-susceptibility phenotype of human STAT2 deficiency has been considered milder than in patients with AR STAT1 deficiency (38,134).This suggest that type I IFN signaling may be not essential for host defense against the majority of common viral infections (135).

8. Cancer immunity

Cancer remains one of the leading causes of death globally; with an estimated 12.7 million cases around the world. The immune system interacts intimately with tumours

over the process of disease development, progression and metastasis. This complex cross talk between immunity and cancer cells can both inhibit and enhance tumour growth (136,137).

The immune system is naturally capable of detecting and eliminating cancer cells through immune surveillance (138–140). Tumour development involves the interplay between cancer cells and host defence mechanisms. Other factors such as infection, chronic inflammation or disease-induced stress may also contribute to tumour growth or tumour suppression (141,142). CTL, NK cells and CD4 Th1 cells are the effector arms of the anti-tumour immune responses, and can stop cancer development via different mechanisms involving the production of IFNγ and cytotoxins (136,143,144).

DCs are the most potent APCs, their interaction with the T cells signal cascades result in the transcriptional activation of genes for T cell differentiation and proliferation. These activated T cells can then act on tumour cells. The engagement of the TCR with the MHC antigen complex on tumour cells triggers lytic granule mobilization and tumour cell lysis. In this context, CTL and NK cells use the same lytic process for the induction of target cell death, but triggering occurs via different receptors (144–146).

8.1 IFNs in Cancer

IFNs regulate the expression of many genes that directly affect tumour cell growth, proliferation, differentiation, survival, migration and other specialized functions. IFNs can target tumour cells directly to inhibit proliferation, alter the cell cycle and induce apoptosis, and activate antitumour immunity (147). Endogenous IFNs has also been shown to modulate the antitumour immune response. The molecular mechanisms associated with the immunoregulatory effects of IFNs include the regulation of tumour antigens on tumour cells as well as antigen presentation by MHC and ligands for receptors of immune checkpoints such as those in the programmed cell death protein 1 (PD1) pathway. Another mechanism of IFN regulation of immunity in cancer is stimulation of the release of secondary mediators such as chemokines, cytokines and interleukins (76,147,148).

The role of type II IFN is known to play a pivotal function on cancer immune surveillance, stimulating antitumor immunity and promoting tumour recognition and elimination (75,149–153). The first reports pointing to the relevance of IFNγ in antitumor immunity came from studies with cancer cell lines. These cell lines, which lack the expression of the IFNγR1 subunit and are refractory to IFNγ signalling, displayed enhanced tumorigenicity compared with control cells, suggesting that IFNγ plays an important role in tumour cell elimination (75). Mouse models showed that IFNγ and lymphocytes are important in reducing the incidence of carcinogen-induced sarcoma and spontaneous epithelial carcinomas (154). Patients with spontaneously

regressing melanoma showed signs of tumour-specific clonal T-cell expansion providing evidence of immune surveillance (138).

Under physiological conditions, the constitutive expression of type I and II IFNs is tightly controlled, remaining localized to tissues (155–157). Upregulation of cell surface MHC class I by IFNγ is crucial for the host response to intracellular pathogens and tumour cells, due to cytotoxic T cell activation, promoting cell-mediated immunity (75). One of the main effects of IFNs is the upregulation of the MHC molecules. Furthermore, in some tumour types, IFNγ can also upregulate the MHC class II transactivator (CIITA) that leads to MHC class II expression (158). Thus, IFNγ initiates an immune-antigenic exposure program in the target cells, and this ensures the rapid recognition of stressed tissues. IFNγ also upregulates cell surface MHC class II on APCs, thus promoting peptide-specific activation of CD4 T cells (159,160). In addition, IFNγ activates macrophages toward a pro-inflammatory profile, inducing polarization toward a tumoricidal phenotype (75).

Separately, IFNγ is involved in antiproliferative, anti-angiogenic and pro-apoptotic effects established against neoplastic cells (75,161–163). The mechanisms by which IFNγ exerts its antitumor effects depend on multiple processes. IFNγ acts as an antiproliferative agent that regulates the expression of cyclin-dependent kinase inhibitor 1 (p21) through STAT1 activation in tumour cells (164). Moreover, IFNγ is able to promote tumour cells apoptosis by upregulating the expression of caspase-1, -3, -8 and by enhancing the secretion of FAS and FAS ligand and tumour necrosis factor (TNF)-

related apoptosis-inducing ligand (165–167). Recent studies showed that IFNγ also induces its tumoricidal effects through a form of regulated necrotic death (168). IFNγ is critical for T cell, NK and NKT cell trafficking into the tumours through chemokine induction (169) and can upregulate intercellular adhesion molecule 1 (ICAM-1) which promotes NK: target cell interaction for an efficient lysis (170).

8.2 Immune evasion in cancer

The immune system is capable of distinguishing between tumour and self, but cancer still develops in immunocompetent individuals. Due to the high mutation frequency of tumours, they can avoid the original immune response but be attacked as a consequence of immune response adaptation. However, eventually the tumour may escape in a process termed "immune editing" and grows despite the immune response (138)**.** During cancer immune editing, the immune system is able to recognize and destroy cancer cells that present tumour antigens. However, constant tumour cell division can generate reduced immunogenicity, enabling tumours to impair the capacity of the immune system to eradicate them by immune suppressive effects or by loss of target antigen expression (136).

8.2.1 Mechanisms of Tumour Immune Evasion

It has been demonstrated that the dysfunction of the host's immune system represents one of the major mechanisms by which tumours evade immunosurveillance. In addition, escape from immunosurveillance can also be linked to tumour-related factors, including

secretion of immunosuppressive cytokines, resistance to apoptosis, and deficient expression of immunomodulatory molecules and MHC class I antigens. Host and tumour-related mechanisms can lead to a failure of the anti-tumour-specific immune response, and these are key factors in the success of cancer immunotherapy (144).

The presentation of antigen in the context of MHC molecules is crucial both during T cell priming and during the effector phase of an adaptive immune response. Alterations in the MHC class I and MHC class II antigen processing and presentation machinery have been demonstrated in different tumours. The MHC class I antigen presentation pathway is disrupted as a consequence of mutations and/or dysregulation of one or several genes (144,171). Down-modulation of the antigen processing machinery and expression of tumour antigens is associated to enhanced tumour incidence and metastasis because CTL can not recognize target antigens on the tumour cells (136,172).

T cell and NK cell responses to tumour antigens can be also diminished by resistance to FAS or TRAIL- induced apoptosis, inhibition of cytotoxic activity via expression of NK cell inhibitory receptors, and a tumour environment not permissive to T cell infiltration (144). Most tumour cells fail to express costimulatory molecules and induce anergy or tolerance in T cells by engaging the T cell receptor in the absence of costimulation. Tumours are also known to evade immune attack by shifting the balance from Th1 to Th2 (immune deviation). Tumour expression of inhibitory molecules like PD-L1 has been shown to cause deletion or anergy of tumour reactive cells (136,173,174). Immune suppression in the tumour microenvironment could be mediated by Tregs through cell-

mediated chemokine production (136). CTL function could be affected through the production of several immunosuppressive cytokines, either by the cancer cells or cells present in the tumour microenvironment (136).

8.3 Defects in the IFN gamma signalling pathway involving cancer susceptibility

Immune evasion can operate through tumour cells losing responsiveness to IFNγ signalling to avoid its antiproliferative, pro-apoptotic, and immunoregulatory actions. This has been demonstrated in tumour cells losing the receptor for IFNγ or a component of JAK/STAT signalling (161). Cellular defects on IFNγR1 and of JAK proteins and may explain the ability of many tumour cells to evade the immune response. Recently, JAK1/2 deficiency was demonstrated to protect melanoma cells from antitumor IFNγ activity and resulting in T-cell-resistant melanoma lesions (75,175).

In addition, somatic mutations in JAK1 were seen in high-risk bladder cancer and gynaecological carcinomas (176,177), supporting the idea that defective JAK1 signalling could play a role in the pathogenesis of some epithelial cancers. LOF mutations in JAK1/2 were associated to resistance to anti-PD-1 therapy in human melanoma cell lines, through the lack of reactive PD-L1 expression and response to IFNγ (178). Acquired resistance to PD-1 blockade immunotherapy has also been described in patients with melanoma, inducing insensitivity to its antiproliferative effects on cancer cells (179). Primary and acquired resistance limit the application of PD-1/PD-L1 blockade therapy (178–180). Regarding the importance of IFNγ in cancer

diagnostics, IFNγ-associated signatures have a predictive value in several cancer immune phenotypes (55,153,154).

8.4 Other roles of IFN in tumour biology

IFNs are cytokines with plethoric functions in different cell types, affecting cell growth, proliferation, differentiation, survival, among other specialized functions (147). IRF1, a member of the IFN regulatory transcription factor family, is an essential transcription factor in the regulation of the cornified envelope genes during keratinocyte differentiation and is a primary response gene in myeloid differentiation. In epithelial cells, IFNs themselves have been shown to modulate keratinocyte differentiation and the expression of genes regulating growth and differentiation (183–185). IFNγ has also been described to induce neuroendocrine (NE)-like differentiation of human prostate basal-epithelial cells (186).

The nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) is highly expressed in different tissues including the developing and mature urothelium (187), and is implicated in the induction of differentiation of normal human urothelial (NHU) cells. Activation of PPARγ in urothelial cells leads to production of the intermediary transcription factors FOXA1 and IRF1, involved in mediating the uroepithelial differentiation programme (188). IRF1 is a member of the IFN regulatory transcription factor family and is one of the major primary response genes induced by IFNγ/STAT1 (75,88). Urothelium is an epithelial barrier tissue and play a role in innate and adaptive immunity through the induced expression of immunoregulatory cytokines

and adhesion molecules (189). The urothelium is prone to chronic inflammatory conditions, epithelial damage and reversal of differentiation. In view of this, it is conceivable that PPARγ-mediated induction of IRF1 may play a dual role in promoting differentiation and modulating inflammation (188).

HYPOTHESIS, JUSTIFICATION AND OBJECTIVES

Hypothesis:

JAK1 is non-redundant for the normal function of human immune cells via cytokine receptor signalling.

Justification:

We have identified a novel immunodeficiency associated with hypomorphic LOF mutations of *JAK1* in a patient with recurrent atypical mycobacterial disease and fatal bladder carcinoma. The purpose of the project is to determine how JAK1 deficiency leads to impaired function of immune cells in humans.

Objectives:

- Aim 1: To generate JAK1-defective cell lines.
- Aim 2: To examine the effect of JAK1 deficiency on signalling through IFN cytokine receptors.
- Aim 3: To investigate the effect of JAK1 deficiency on mycobacterial susceptibility.
- Aim 4: To investigate the importance of JAK1 in anti-viral protection.
- Aim 5: To investigate the importance of JAK1 in cancer susceptibility.

MATERIALS AND METHODS

Patients and human cell lines

THP1 cells from American Type Culture Collection (ATCC #TIB-202) were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin-streptomycin (P/S). Patient and control fibroblasts as well as 293T cells were cultured in DMEM medium and 10% heat-inactivated FCS and 1% P/S. For the different experiments fibroblasts were detached using Accutase® solution (A6964, Sigma Aldrich). Regular checks for *Mycoplasma spp* contamination were performed. JAK1-complented stable clones were kept in the presence of puromycin (3ug/ml). Patient and healthy control Epstein-Barr Virus (EBV)-immortalized B cell lines were derived from peripheral blood mononuclear cells (190). Informed written consent was obtained in accordance with the Declaration of Helsinki and ethical approval from the Great Ormond Street Hospital for Children NHS Foundation Trust and the Institute of Child Health Research Ethics Committee (Reference Number: 06/Q0508/16).

Normal and immortalised human urothelial cell culture

Blood samples and biopsies were obtained with ethical approval (National Research ethics numbers 08/H0720/46, 99/095 and 02/208) and informed consent from all subjects in accordance with the Declaration of Helsinki. Normal human urothelial (NHU) cells obtained ethically with appropriate informed consent and Research Ethics Committee approvals were maintained in vitro as non-immortalized (finite) cell lines, using protocols detailed in full elsewhere (191). For routine culture, NHU cells were

grown as adherent monolayers on Primaria™ plasticware (BD Biosciences) in low calcium (0.09 mM) keratinocyte serum-free medium (KSFM) containing bovine pituitary extract and recombinant epidermal growth factor (Life Technologies) supplemented with 30 ng/ml cholera toxin (KSFMc). NHU cell lines were sub-cultured by trypsinisation at just-confluence and used in experiments between passages 3-5. Experiments described here were performed on five independent NHU cell lines. Due to the finite nature of these lines, no genotyping of individual cell lines was performed. Differentiation of NHU cells was induced in just-confluent cell cultures using 1 μM troglitazone (TZ) as PPARγ ligand with concurrent 1 μM PD153035 to block epidermal growth factor receptor (EGFR) activation, as previously described (192). An immortalized NHU subline produced by retroviral transduction with human telomerase reverse transcriptase (hTERT) cells as detailed elsewhere (193), was used in this study. The line, referred to as Y235hTERT, was previously characterized at passage 40 against the pre-immortalised parental line (passage 7) using comparative genomic hybridization. The Y235hTERT cells for this study were used within 20 passages of the comparative genomic hybridization (CGH) analysis. JAK1 knock down (KD) and scrambled control (Sc) hTERT urothelial cell lines were generated using lentiviral vectors expressing short hairpin RNA (shRNA) sequences. Cultures were tested regularly for contamination by *Mycoplasma spp.* using polymerase chain reaction-based kits and DNA-intercalating fluorescent stains for presence of extranuclear DNA.

Lentivirus preparation and transductions

JAK1 knock down (KD) and scrambled control (Sc) hTERT urothelial cell lines were generated using lentiviral vectors expressing short hairpin RNA (shRNA) sequences.

pGIPZ vectors carrying the short hairpin RNA against JAK1(TAGTACACACATTTCCATG) or scrambled control (TGAACTCATTTTTCTGCTC) sequences as well as puromycin resistance cassette and turbo-GFP marker for selection were supplied by University College London Open Biosystems (London UK). Lentivirus stocks were prepared by transfection of 293T cells (80-90% confluence) cultured in DMEM medium and 10% heat-inactivated fetal bovine serum, with the envelope plasmid 17.5ug pMD.G2 (VSV-G/envelop), 32.5ug p8.74 plasmid (gag-pol) and 25ug vector construct with the transfection reagent PEI/Optimen following the manufacture instructions. Medium was replaced 5h post transfection and medium was harvested after 24 and 48 hours, cleared by centrifugation (4000 rpm, 5 min), filtered through 0.22-µm filters and left to spin for 2h 4ºC 50,000g. Viruses were tittered on 293T cells by scoring green fluorescent protein (GFP) positive cells by flow cytometry 3 days post transduction. Virus stocks were stored at -80°C. Transductions of the cells were carried out by infection at a multiplicity of infection of 1:10 for 6h, and then the virus containing media was replaced by fresh media. Cells were selected in puromycin-containing medium (3ug/ml for THP1 cells and 1ug/ml for hTERT urothelial cell) and the efficiency of transduction was assessed as percentage of GFP positive cells by flow cytometry. Lack of JAK1 expression in JAK1-deficient cells was verified by reverse transcription polymerase chain reaction *(*RT-PCR).

Determination of mRNA levels by real time-quantitative polymerase chain reaction (RT-qPCR) and reverse transcription polymerase chain reaction (RT-PCR)

Cells were left unstimulated or stimulated with the given concentrations of IFNγ (Invitrogen) or IFNα (Roche) for different time points (detailed in the legends). Total RNA from cells was extracted using RNAeasy kit (Qiagen). RNAs were converted to cDNA by reverse-transcription using Quantitect reverse transcription kit (Qiagen). Determination of mRNA level was performed by RT-PCR using specific primers (table S1) and QuantiTect SYBR® Green PCR Kit (Qiagen) according to manufacturer's instructions. Fold changes were calculated using the DDCT2 (-Delta Delta C(T)) method and results were normalized with respect to the values obtained for the endogenous Actin and GAPDH cDNA.

Table 3. Primers used for RT-qPCR and RT-PCR

Target	Forward (5' → 3')	Reverse (5' → 3')
JAK1	TGGATCTCTTCATGCACCGGA	ATGAATGGGCCACACTCACTG
IRF1	CAGAGAAAAGAAAGAAAGT	CATCAGAGAAGGTATCAG
CIITA	ATG CGC TGA GTG AGA ACA AGA TC	GGAAGCGGAGGTGAGGAGATT
MX1	TCACCAGAGAATAACAGAGG	GGCATTAACTTTATCTATCAGG AA
GAPDH	GAGCCACATCGCTCAGACAC	CATGTAGTTGAGGTCAATGAA GG
Beta-Actin	CAGCAAGCAGGAGTATGACG	AAAGCCATGCCAATCTCATC
FOXA1	CAAGAGTTGCTTGACCGAAAGTT	TGTTCCCAGGGCCATCTGT
PPARγ	GAACAGATCCAGTGGTTGCAG	CAGGCTCCACTTTGATTGCAC

RT-qPCR= Real time quantitative polymerase chain reaction, RT-PCR= Reverse transcription polymerase chain reaction, JAK1= Janus associated kinase 1, IRF1= Interferon regulatory factor 1, GAPDH= glyceraldehyde-3-phosphate dehydrogenase, FOXA1= Forkhead box protein A1, PPARγ= Peroxisome proliferator-activated receptor gamma.

Surface staining and analysis of STAT1 phosphorylation Flow Cytometry

For surface staining, Sc and KD hTERT urothelial cells, +/- addition of the given concentrations of IFNγ (detailed in the legends) for different time points, were detached using Accutase® solution (A6964, Sigma Aldrich), labelled with fluorescent-conjugated antibodies (see Table S2) and washed with phosphate buffered saline (PBS). For STAT1 phosphorylation analysis, cells were stimulated with the given concentrations of

IFNγ or IFNα (detailed in the legends) for 10 min, or not stimulated, then fixed and permeabilized using fix buffer I and Perm Buffer III (BD Biosciences) for 30 min at 4ºC, washed with PBS and labelled with 5µL anti-pSTAT1 antibody (pY701. BD Biosciences) for 60min in the dark. For all flow cytometry (BD LSRFortessa) experiments 10,000-30,000 gated events were collected and analysed using FlowJo software.

Table 4. Antibodies used for flow cytometry

Antibody	**Catalog No.**	**Supplier**
anti-HLA-ABC (FITC)	IM1838U	Beckman Coulter Immunotech
anti-CD119 (IFNGR1) (PE)	558937	BD Bioscience
anti-CD54 (ICAM-1) (APC)	559771	BD Bioscience
anti-CD274 (PD-L1) (PE)	329705	BioLegend
anti-HLA-DR (PerCP)	347402	BD Bioscience
Anti-STAT1 (pY701) (Alexa Fluor 467)	612557	BD Bioscience

HLA= Human leukocyte antigen, IFNGR= Interferon gamma receptor, ICAM-1= Intracellular adhesion molecule 1 (CD54), PD-L1= Programmed death-ligand 1, STAT1= Signal transducer and activator of transcription, FITC= Fluorescein-5-isothiocyanate, PE= Phycoerythrin, APC= Allophycocyanin, PerCP= Peridinin-Chlorophyll-protein.

Infection models with bacteria in vitro

The *Mycobacterium bovis* Calmette–Guérin (BCG) Pasteur strains (ATCC® 35748™), BCG expressing-mCherry (kind gift from Prof. Brian Robertson Imperial College of London, London, U.K) and *Salmonella typhimurium* (ATCC 14028) (kind gift from Dr Dagmar Alber, UCL, London, U.K) were used in the study. Mycobacteria were grown

to mid-log phase (optical density (OD) between 0.6-1) in Middlebrook 7H9 medium supplemented with 10% OADC enrichment medium (BD Biosciences), plus 50 μg/ml hygromycin for BCG expressing-mCherry. Stock cultures were maintained in glycerol at –80 C until later use. Viable cell counts in thawed aliquots of BCG were determined by plating serial dilutions of cultures onto supplemented Middlebrook 7H11 agar plates followed by incubation at 37ºC for 14-21 days. Salmonella was grown to mid-log phase in LB broth overnight with agitation.

THP1 cells were differentiated in macrophages using 10ng/ml of phorbol myristate acetate (PMA) for 48h and then were left unstimulated or stimulated with IFNγ 50ng/ml for 18h before infection. Cells were infected using stocks (BCG) or bacteria in mid-log grow phase (salmonella), using a multiplicity of infection (MOI) of 20:1 for BCG expressing-mCherry and 10:1 for BCG and salmonella. The MOI calculation was performed using the following conversion: OD of 1 = 1 x 10^8 colony forming units (cfu)/ml for BCG and OD of 1 = 10^9 cfu/ml for salmonella. Bacteria were washed and suspended in RPMI medium and 10% heat-inactivated FCS. Monolayers were incubated for 4 h with BCG and 30 min with salmonella at 37ºC in 0.5% CO2. Infected cells were washed to remove extracellular bacteria. Cells were also incubated in complete medium with gentamicin (100μg/ml) for two hours after salmonella infection, in order to kill extracellular bacteria. Subsequently, macrophages were incubated in fresh complete medium, in the presence or absence of IFNγ (50 ng/ml) for different time points (detailed in the legends).

Harvest of infected macrophage lysate for cfu plating

Cells were lysed at 24h for salmonella and 3 days for BCG infection with 0.05% SDS w/v in H2O and serial dilutions were plated out on Middlebrook 7H11 agar plates followed by incubation at 37ºC for 14 days for BCG infection. After salmonella infection, serial dilutions were plated out on LB agar plates to count the number of cfu/ml after 12hr at 37ºC.

Quantification of the infected cells by Flow Cytometry

Macrophages differentiated from the scrambled control and KD THP1 cell lines using PMA, were left unstimulated or stimulated with IFNγ (50ng/ml) before infection with BCG expressing-mCherry strains. After phagocytosis, cells were washed and incubated in complete medium in the presence or absence of IFNγ (50 ng/ml) for the given time points. Cells were removed from the plate using Accutase® solution (A6964, Sigma Aldrich), washed with PBS, fixed in 4% paraformaldehyde (PFA) for 10 min and analysed by flow cytometry (BD LSRFortessa) using FlowJo.

Microscopy

200,000 THP1 cells were differentiated on 35mm glass bottom dishes (Fluorodish). After phagocytosis of BCG expressing-mCherry, cells were washed and incubated in complete medium in the presence or absence of IFNγ (50 ng/ml) for the given time points. Subsequently, cells were incubated with 50nM LysoTracker Deep Red (Life Technologies) for 30min, washed and then fixed in 4% paraformaldehyde (PFA) for 10 min. Nuclei were stained with 5µg/ml DAPI for 10 min, cells were then washed and kept on PBS. Cells were acquired using Leica inverted fluorescent microscope equipped

with 60x oil objective for quantification of infected cells or Nikon Eclipse Ti-E confocal microscope equipped with 40x objectivefor colocalisation analysis. Infected cells were counted manually and at least 100 cells per experiment were analysed. Images were processed using ImageJ (National institute of health) and Imaris image analysis software.

pH sensitivity of pHrodo-labelled BCG

BCG-lux were labelled with pHrodo™ (Invitrogen) at a concentration of 25mM according to the manufacturer's instructions, except for omission of the 100% methanol step. Approximately 100,000 cfu were resuspended in 500 µl buffer at pH 7. Samples were then acquired on a BD Fortessa flow cytometer (BD LSRFortessa), and pHrodo fluorescence was measured in the PE-Texas Red channel, and analysed using FlowJo.

Cell viability and Apoptosis assays

Sc and KD hTERT urothelial cells were stimulated with IFNγ using different time points and concentrations. Alamar Blue® (AB), diluted 1:10 with KSFMc, was added to urothelial cells grown in 96-well plates (5,000 cells/100ul). After 3 hours incubation at 37°C, absorbance was measured at 560 and 620 nm. AB reduction was calculated according to manufacturer's instructions (AbD Serotec, Kidlington, UK).

Apoptosis was determined by flow cytometry using APC Annexin V apoptosis detection kit with Propidium Iodide (PI) according to manufacturer's instructions (BioLegend 640932). For apoptosis assays, macrophages differentiated from scrambled

control and JAK1-deficient THP1 cell lines were left unstimulated or stimulated with the given concentrations of IFNγ (detailed in the legends) before BCG infection (MOI 10:1); and then incubated in complete medium in the presence or absence of IFNγ for the given time points (detailed in the figure legends). Sc and KD hTERT urothelial cells were left unstimulated or stimulated with the given concentrations of IFNγ (detailed in the legends). Percentage of apoptosis was determined using APC Annexin V apoptosis detection kit with PI (BioLegend 640932) according to manufacturer's instructions by flow cytometry (BD LSRFortessa), and analysed using FlowJo.

Viral assays

For the plaque assays, control and patient fibroblasts were grown in six-well dishes and infected with different dilutions of PIV5VΔC and PIV5 for 1h. Subsequently, 0.1% Avicel (FMC Biopolymer) was included in the overlay medium and cells were incubated for 5 days at 37ºC in 0.5% CO2. Plaques were visualized by immunostaining using a pool of monoclonal virus-specific antibodies for the viruses as described previously (194,195), together with alkaline phosphatase-conjugated secondary antibody by using SIGMAFAST BCIP/NBT as the substrate.

Control and patient fibroblasts were grown on 13 mm diameter coverslips in individual wells of 24-well plates and then were left unstimulated or stimulated with different concentrations of IFNγ or IFNα overnight. Cells were infected with PIV5 at a multiplicity of infection of 10pfu/cell. The inoculum was adsorbed for 1 h and then cells were incubated in complete medium in the presence or absence of IFN for 24h.

Monolayers were incubated in fixing solution (5% formaldehyde and 2% sucrose in PBS) for 15 min at room temperature, then permeabilized (0.5% Nonidet-P40 and 10% sucrose in PBS) for 5 min, and washed three times in PBS containing 1% FCS and 0.1% azide (PBS, 1% FCS, 0.1% azide). To detect the proteins of interest, cell monolayers were incubated with 10–15 μl of an antibody used to detect PIV5 (196). Cells were subsequently washed (PBS, 1% FCS, 0.1% azide). In addition, cells were stained with the DNA-binding fluorochrome DAPI (0.5 μgml−1; Sigma-Aldrich) for nuclear staining. Following staining, monolayers were washed with PBS, mounted using Mowiol and examined using a Nikon Microphot-FXA immunofluorescence microscope.

EBV-B cells viral assays were performed as previously described (134). EBV–B cells were either left untreated or were treated with 10,000 IU/ml IFNα for 18 h. The kinetics of VSV growth in EBV-B was determined by resuspending the cells in RPMI medium containing the virus inoculum and incubating for 1 h (VSV MOI = 1), washing with PBS and resuspending in fresh complete medium. Virus-containing supernatants were then collected at the indicated time points. VSV titers were determined by calculating the 50% end point (TCID50), as previously described (197), after the inoculation of 96-well plates with Vero cell cultures.

Lymphocyte Cytotoxicity Assay

Healthy donor peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and frozen in 10% dimethyl sulfoxide (DMSO). To control for differences in

the frequency of lymphocyte subsets between donors, PBMCs were collected from a single donor buffy coat and used for all killing assays shown. PBMC were thawed and re-suspended in RPMI (Invitrogen) with 10% heat-inactivated fetal bovine serum and monocytes removed by plastic adherence (1h, 37 °C). Urothelial cells were cultured in 96-well plates +/- IFNγ (5 ng/ml) for 30h and subsequently co-cultured overnight with interleukin-2 (IL-2) (Roche) + monocyte-depleted PBMCs (50:1). Cells were detached using Accutase® solution, washed with PBS and resuspended in 200 µl DNA staining solution (NKTEST, Glycotope Biotechnology). Urothelial cells were gated based on GFP expression and analysed by flow cytometry.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 5.1 Software. Associations between JAK1-deficient and control cells were tested using one-way ANOVA and appropriate post-test, or a two-tailed Mann Whitney U test. A p value of <0.05 was considered significant.

RESULTS

Chapter I – Role of partial JAK1 deficiency in mycobacterial susceptibility

Description of the patient with JAK1 deficiency

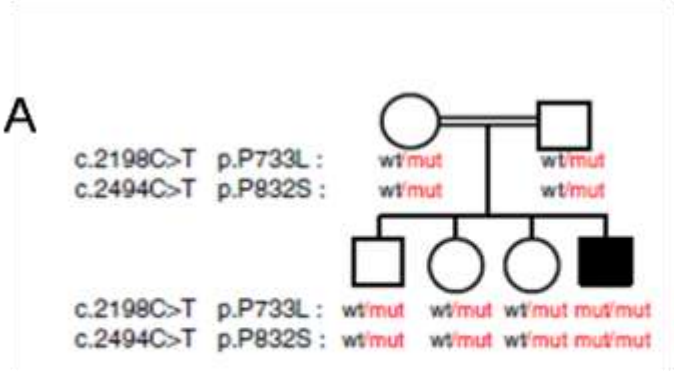
A 23-year-old male from Pakistan was born from a consanguineous marriage. The patient presented at the age of 3 years with a history of recurrent respiratory tract infections, cervical lymphadenopathy and developmental delay. The patient had received childhood vaccines including BCG at birth and had normal-course chicken pox at age 3 with one subsequent episode of shingles. A skeletal survey was performed demonstrating lytic lesions affecting long bones, vertebrae and facial bones. Biopsies were unremarkable and no pathogens were isolated from either tissue. Mycobacterial skin tests were performed, showing strongly positivity for *Mycobacterium malmoense* and *Mycobacterium scrofulaceum.* In view of these findings, the patient received treatment for systemic atypical mycobacterial infection, resolving the multifocal osteomyelitis after 12 months

The immunology investigations demonstrated normal numbers of T and B cells, reduced naive CD4 and CD8 T cells and normal proliferation after phytohemagglutinin (PHA) stimulation, normal immunoglobulin levels and specific antibody responses after vaccinations. Over the time, the IgM levels fell below the normal range with persistent mild T-cell lymphopenia and impaired responses to PHA stimulation. At the age of 16 presented with cardiomyopathy and computed tomography imaging revealed a mediastinal mass. Biopsies showed pleural and mediastinal fibrosis with patches of macrophage infiltration in lung tissue. No granulomas were seen and Quantiferon TB

Gold test was negative. In view of his previous history he received empiric treatment for atypical mycobacteria with reduction of the mass. He had no recurrence or further mycobacterial infections on long-term antibiotic prophylaxis. However, he had a number of skin infections, including planar warts in the forehead, fungal infections of his nails and severe Norwegian scabies.

At the age of 21 years the patient developed significant anaemia. Thickening of the bladder wall was noted on magnetic resonance imaging and cystoscopy showed an extensive fungating tumour. Biopsies of the tumour and lymph nodes confirmed a high-grade metastatic transitional cell carcinoma. The patient received treatment with chemotherapy, but died at the age of 23 years.

Exome sequencing revealed two separate homozygous *JAK1* missense mutations that were predicted to be probably damaging, leading to amino-acid changes from proline to leucine (p.P733L) and from proline to serine (p.P832S). STAT phosphorylation was tested in the patient's lymphocytes, observing significantly reduced but not abolished STAT3, STAT5, STAT6 and STAT1 phosphorylation after IL-2, IL-4, IL-10, IFNα and IFNγ stimulations indicating a broad impact on cytokine signalling (Fig. 1). In cell lines models, both mutations were demonstrated to contribute to full JAK1 function, with the P733L mutation conferring the larger loss of function effect (2) (Appendix). In summary, hypomorphic recessive germline *JAK1* mutations induced a functional partial JAK1 deficiency, affecting multiple signalling pathways that clinically manifested with atypical mycobacterial infections and increased susceptibility to cancer (2).


A
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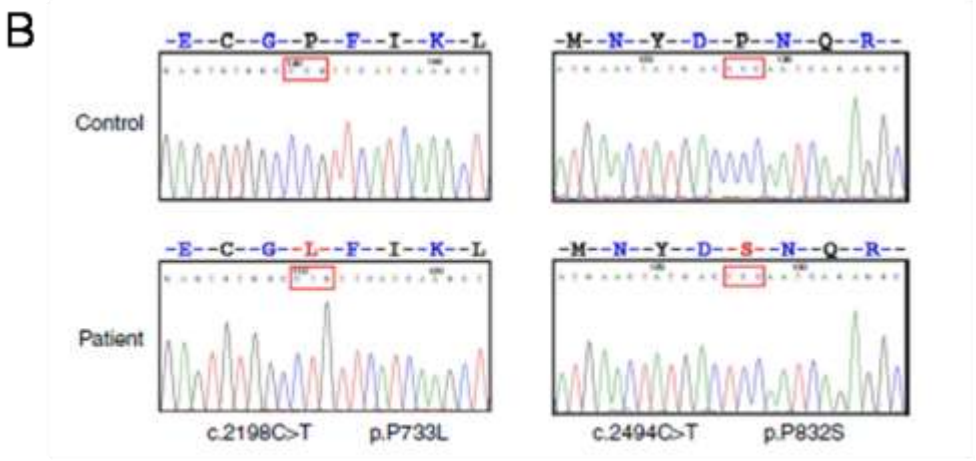
B
-E--C--G--P--F--I--K--L
-M--N--Y--D--P--N--Q--R--
Control
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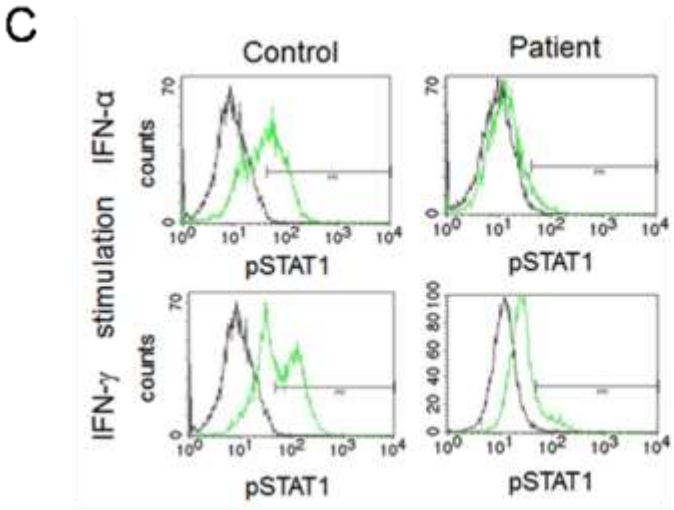
Patient
c.2198C>T p.P733L
c.2494C>T p.P832S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IFN-α stimulation
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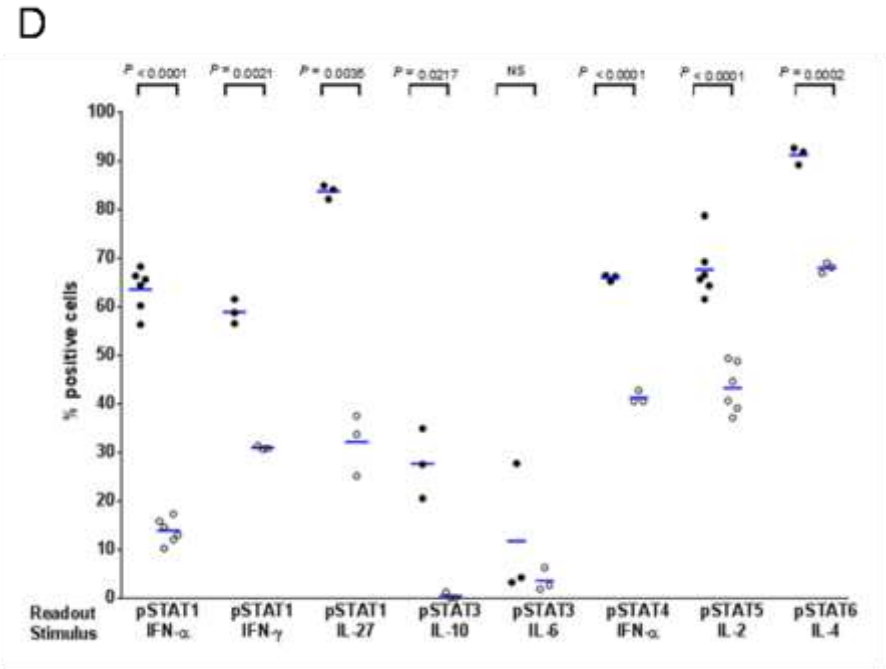
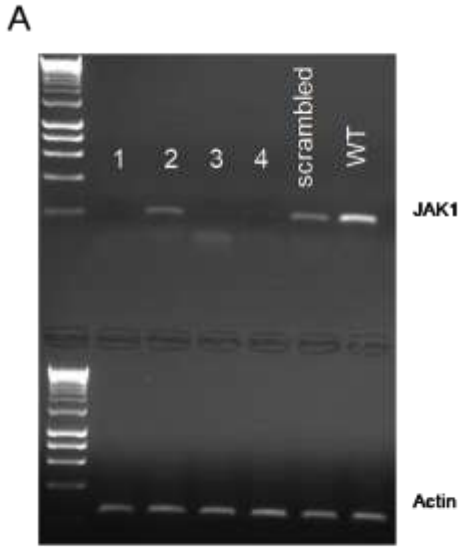


Figure 1. *JAK1* mutations and functional defects found in the patient. (A) Patient's family tree. (B) Sequence chromatograms showing two mutations in JAK1 gene. (C) Flow cytometry gating for pSTAT1 after IFNα and IFNγ stimulation is shown. (D) Percentage of cells positive for the presence of phosphorylated STAT proteins were measured by flow cytometry after 10 min stimulation of whole blood of the patient (open circles) and compared with a healthy control (black dots). Blue lines show geometric means. Unpaired two-tailed Student t-test with Welch's correction. (Adapted from Eletto *et al*. Nature communications. 2016. (Appendix))

Partial JAK1 deficiency impairs STAT1 phosphorylation and expression of IFNγ-inducible genes in THP1 cells.

As our patient with hypomorphic JAK1 deficiency presented predominantly with MSMD, we sought to establish a model to examine the role of JAK1 in myeloid cells during mycobacterial infection. As patient blood was not accessible, for this study we generated a THP1 myeloid cell line with sub-total *JAK1* KD to mimic partial JAK1 deficiency using lentiviral vectors expressing shRNA sequences. Compared to control shRNA JAK1 shRNA substantially reduced JAK1 messenger RNA expression for 3 out

of 4 hairpins tested (Fig.2A). THP1 cells transduced with JAK1 shRNA #3 were utilised for further studies. Demonstration of JAK1 knock down at the protein level by immunoblotting was unsuccessful using several antibodies (including the one used in Eletto *et al)*. To test whether the level of mRNA reduction was sufficient to impair JAK1 protein function, we studied JAK1-mediated activation of STAT1 in response to IFNγ stimulation, using flow cytometry. We observed a significant decrease in STAT1 phosphorylation following IFNγ stimulation in the KD cell line compared to untransduced and scrambled control shRNA lines ($p<0.05$) (Fig.2B,C). STAT1 phosphorylation in response to IFNα stimulation also showed a trend towards reduction although this did not reach significance (Fig.2D,E), suggesting that partial loss of JAK1 function affects predominantly the type II IFN response in this model. Following stimulation with IFNγ, upregulation of *IRF1* and *CIITA* mRNA was significantly lower in the KD than Sc lines, indicating impaired downstream gene transcription in JAK1 deficiency (Fig.2F,G).



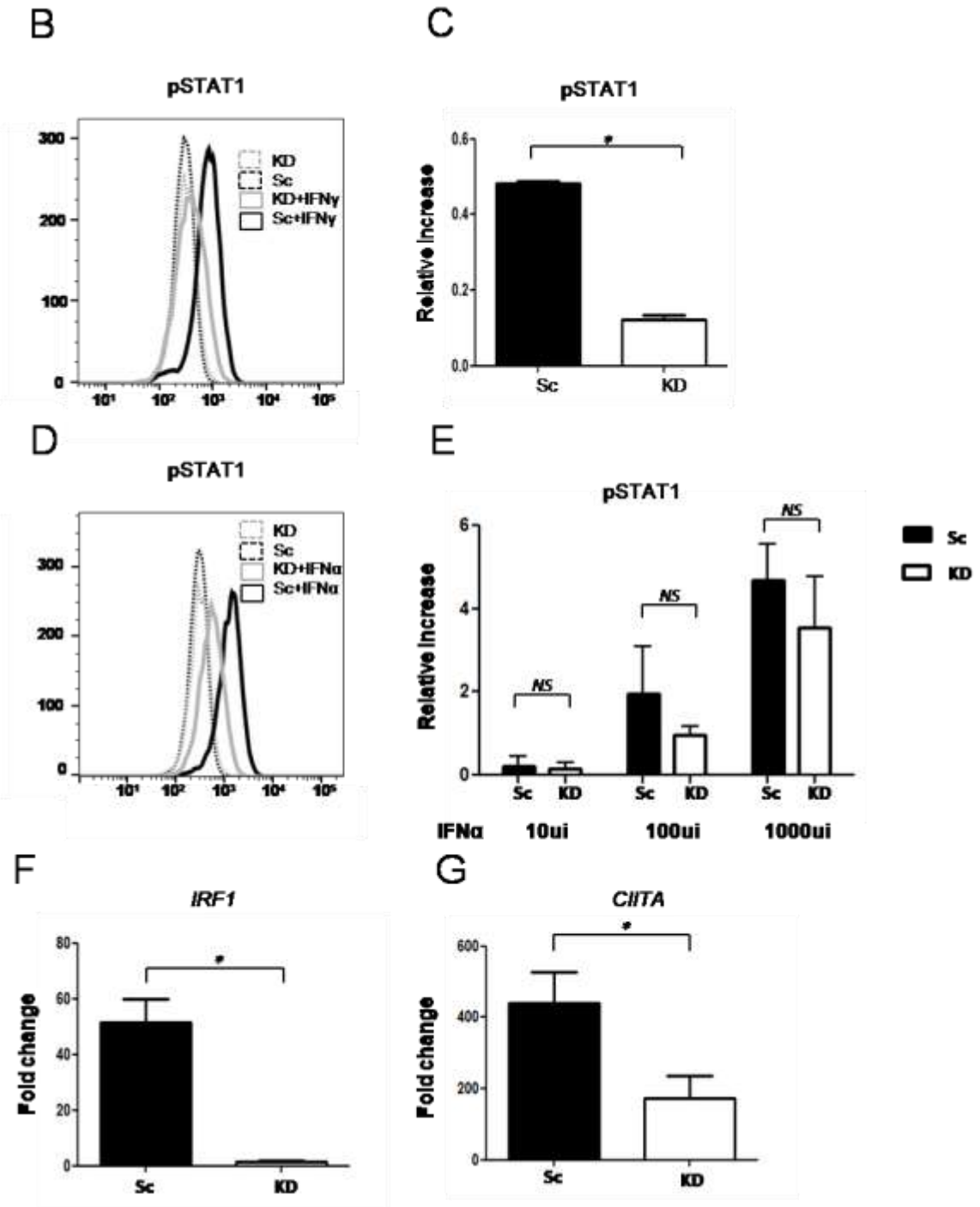


Figure 2. STAT1 phosphorylation and expression of IFNγ inducible genes is impaired in JAK1-deficient THP1cells.

(A)RT-PCR analysis of JAK1 expression in THP1 WT and transduced cell lines (constructs shRNA 1, 2, 3, 4 and Scrambled control). Data is representative from two independent experiments. (B,C) Analysis of JAK/STAT signalling by flow cytometry in

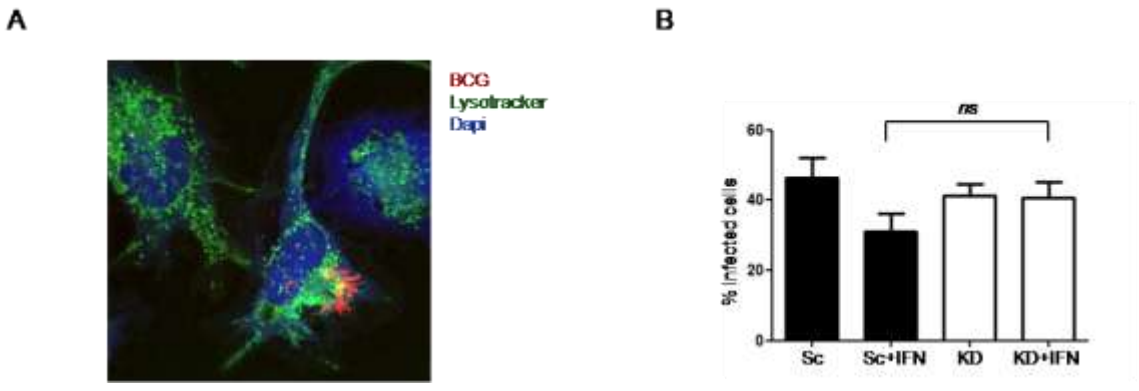
Sc and KD THP1 cells after stimulation with IFNγ (50 ng/ml) for 10min. (D,E) Analysis of JAK/STAT signalling by flow cytometry in Sc and KD THP1 cells after stimulation with different concentrations of IFNα for 10min. B and D display a representative experiment, C and E are from three independent experiments. Two-tailed Mann Whitney test. (F,G) RTqPCR analysis of *IRF1* and *CIITA* expression from KD and Sc THP1 cells after stimulation with IFNγ (50ng/ml) for 24h. Data is from four independent experiments. Two-tailed Mann Whitney test. Graphs show mean values ± SE and regulation of the expression compared to untreated. *P <0.05. Error bars represent the SE.

Partial loss of JAK1 function promotes mycobacterial and salmonella survival in myeloid cells

To test the impact of reduced JAK1 function on IFNγ-mediated host defense to intracellular pathogens we utilized BCG as a well-established model for mycobacterial infection. Macrophages differentiated from the JAK1 KD and Sc cell lines were infected with BCG, with or without prior IFNγ stimulation. THP1 cell lines were capable of internalizing BCG, as seen by confocal microscopy (Fig. 3A). Three days after BCG infection, a trend towards a lower percentage of infected cells was observed in the Sc cell lines after IFNγ stimulation (Fig.3B). In contrast, no difference was seen in JAK1 KD lines in response to IFNγ (Fig. 3B). Using confocal analysis and a lysotracker dye which increases fluorescent intensity in low pH (198), both Sc and JAK1 KD cell lines were observed to traffic a proportion of internalised BCG into acidified compartments (Fig. 3C). It was not possible to compare the number of intracellular BCG by confocal analysis because of bacterial clumping.

Therefore, to better quantitate BCG infection, cells were co-cultured with mCherry-expressing BCG and analyzed by flow cytometry. Similar levels of bacteria were

internalized by KD and Sc lines at 4 hours and this was largely unaffected by IFNγ stimulation (Fig. 3A,B), indicating that loss of JAK1 does not significantly impact phagocytosis. As expected, at both 24 and 72 hours, IFNγ stimulation significantly reduced mCherry fluorescence in Sc lines consistent with lower bacterial survival. In contrast, IFNγ had no significant impact on mCherry levels in KD lines (Fig, 4A,B). To confirm that JAK1 deficiency promotes intracellular BCG survival, KD and Sc lines were lysed on culture plates after infection and surviving bugs quantitated by counting colony forming units (cfu/ml). As seen in flow cytometry assays, BCG survival was higher in KD lines indicating an important role for JAK1 in controlling mycobacterial infection (Fig. 4C). Similar findings were obtained with *Salmonella typhimurium* (Fig. 4D), another intracellular pathogen known to require IFNγ signaling for control of the bacterial infection, with significantly higher bacterial survival seen in KD than Sc cells. Together these results demonstrate that JAK1-deficient myeloid cells permit enhanced intracellular mycobacterial and salmonella survival in vitro.



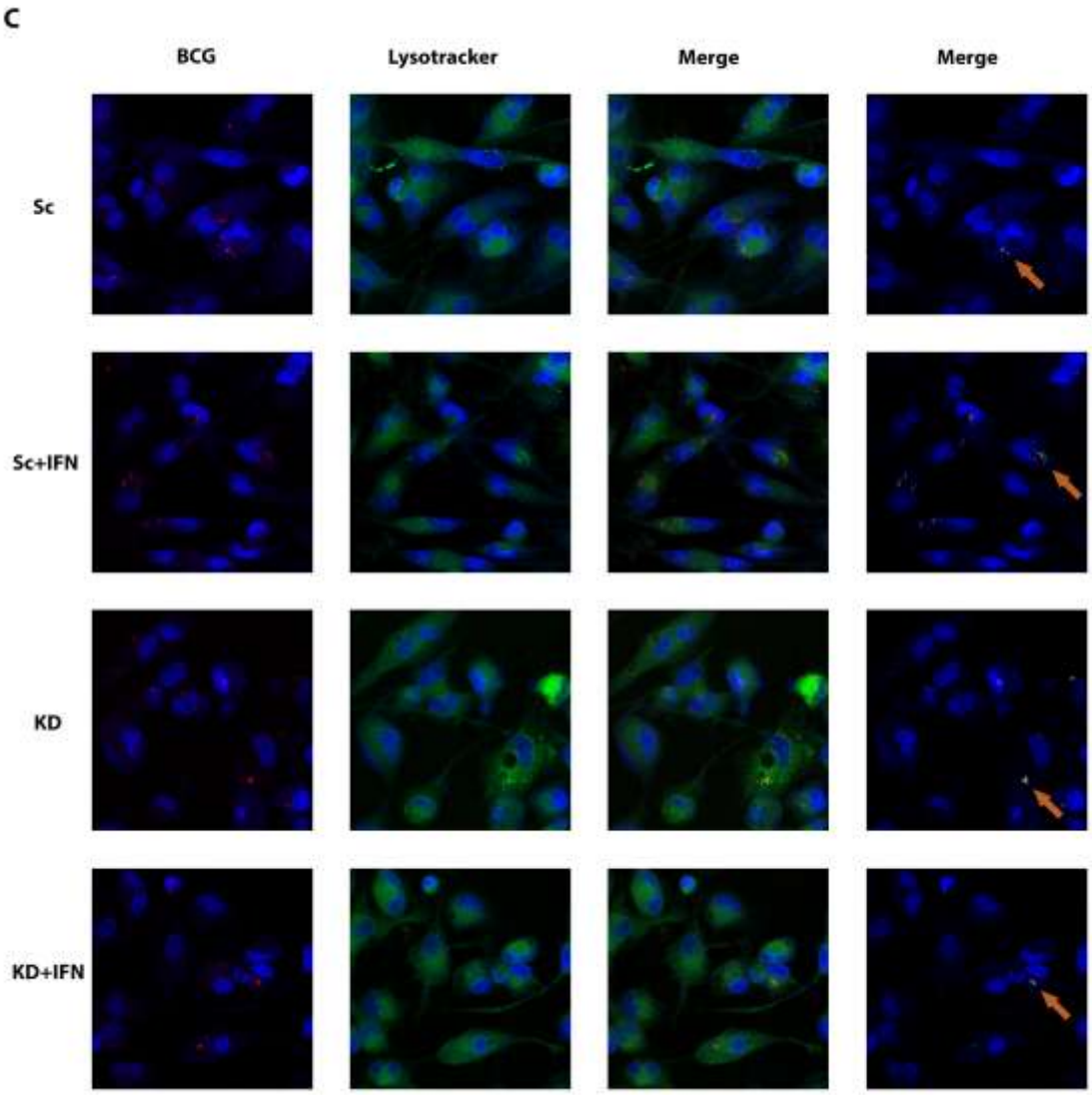


Figure 3. BCG is internalised and localised into acidified compartments in control and JAK1-deficient THP1 cells

(A, B) Internalisation of mCherry-BCG by macrophages differentiated from the scrambled control and KD THP1 cell lines, unstimulated or stimulated with IFNγ (50ng/ml). (C) Acidified phagosomes containing mCherry-BCG (arrow). A and C display a representative experiment, B is from 3 independent experiments.

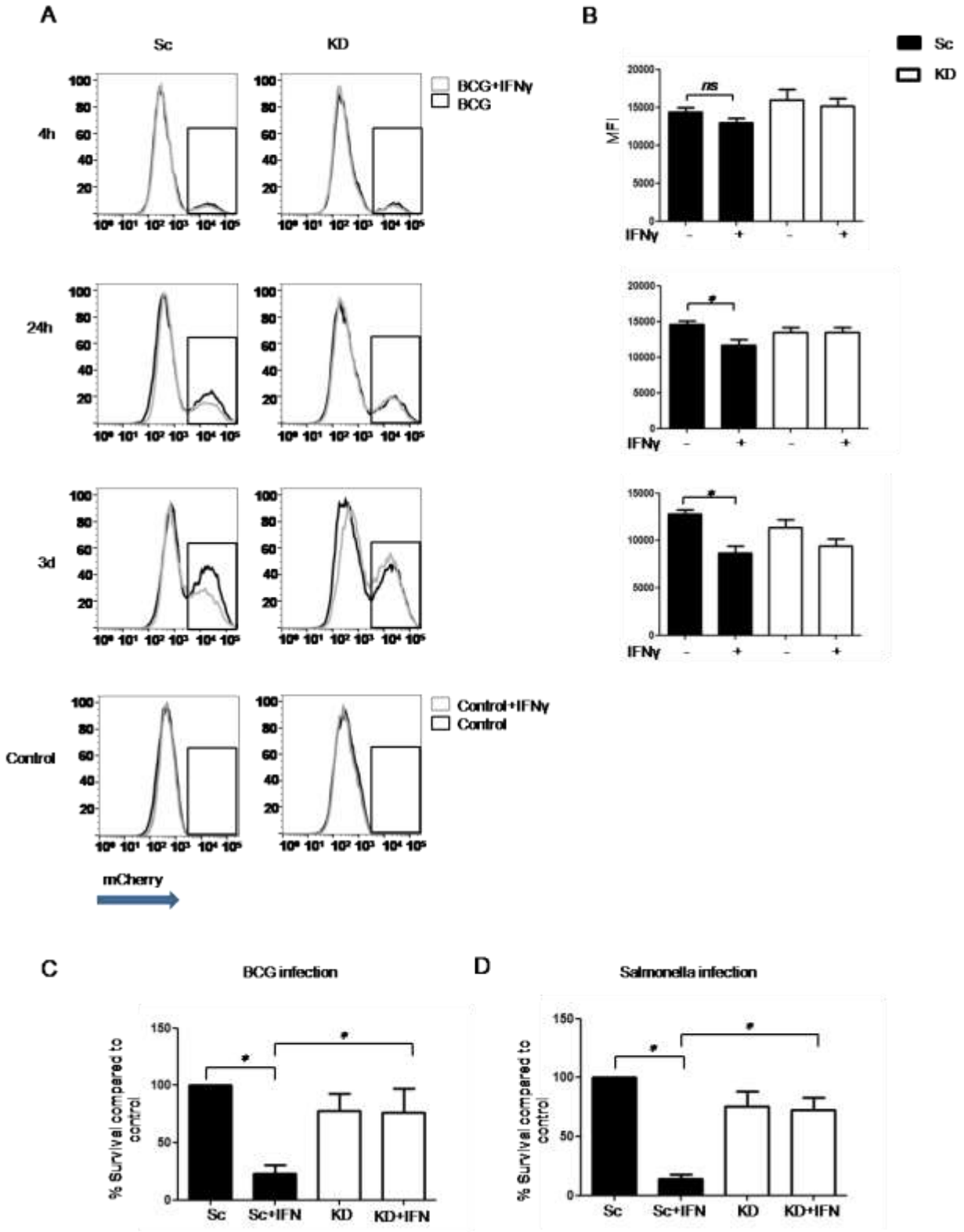

A
Sc
KD
BCG+IFNγ
BCG
4h
24h
3d
Control
Control+IFNγ
Control
mCherry
B
Sc
KD
MFI
ns
IFNγ
C
BCG infection
% Survival compared to control
Sc
Sc+IFN
KD
KD+IFN
D
Salmonella infection
% Survival compared to control
Sc
Sc+IFN
KD
KD+IFN

Figure 4. JAK1-deficient THP1 cells show increased mycobacterial and salmonella survival after IFNγ stimulation.

(A,B) Flow cytometry quantitation of mCherry-BCG in macrophages differentiated from the Sc and KD THP1 cell lines using PMA, with or without prior IFNγ (50ng/ml) stimulation; mCherry fluorescence was measured in the PE-Texas Red channel. Black line – BCG infected cells, gray line – BCG infected cells + IFNγ stimulation. A displays a representative experiment, B is from five independent experiments. (C,D) Bacterial survival in macrophages differentiated from Sc and KD THP1 cell lines, infected with BCG or salmonella strains, with or without prior IFNγ (50ng/ml) stimulation. Data is from six and four independent experiments respectively. Two-tailed Mann Whitney test. *P <0.05. Error bars represent the SE.

Partial JAK1 deficiency impairs IFNγ-induced phagosome acidification and apoptosis in myeloid cells.

To further explore the mechanisms promoting enhanced bacterial intracellular survival in myeloid cells with reduced JAK1 function, phagosome acidification and apoptosis were tested as these are key IFNγ-dependent steps in the control of mycobacterial infection (102,103,114,199–201). Following infection of THP1 cell lines with pHrodo-labelled BCG, phagosomal acidification was measured by measuring fluorescence which is released in the context of low pH. Even in the absence of IFNγ stimulation, both Sc and KD THP1 cells had relatively high levels of pHrodo fluorescence (Fig. 5A,B). Fluorescence intensity was increased after IFNγ-stimulation in Sc cells lines consistent with additional IFNγ-mediated induction of acidification (Fig. 5A-C). In contrast there was no increase in the KD cell line following IFNγ stimulation.

To test whether partial JAK1-deficiency is sufficient to impair IFNγ-induced apoptosis Annexin V/PI staining was measured by flow cytometry. Sc control and KD THP1 cells had similar baseline levels of apoptosis which was not significantly increased 5 days after BCG infection alone (Fig. 5D-F). In contrast, IFNγ pre-treatment of Sc control

cells induced significant apoptosis compared with untreated control cells at both 3 and 5 days after BCG infection (Fig. 5D-F). Significantly less apoptosis was seen in KD cells 3 and 5 days after BCG infection following IFNγ pre-treatment, compared with control cells ($p < 0.05$) (Fig. 5D-F). Together our data suggest that defective intracellular bacterial killing in myeloid cells with reduced JAK1 function is at least in part due to impaired IFNγ-induced phagosome maturation and apoptosis.

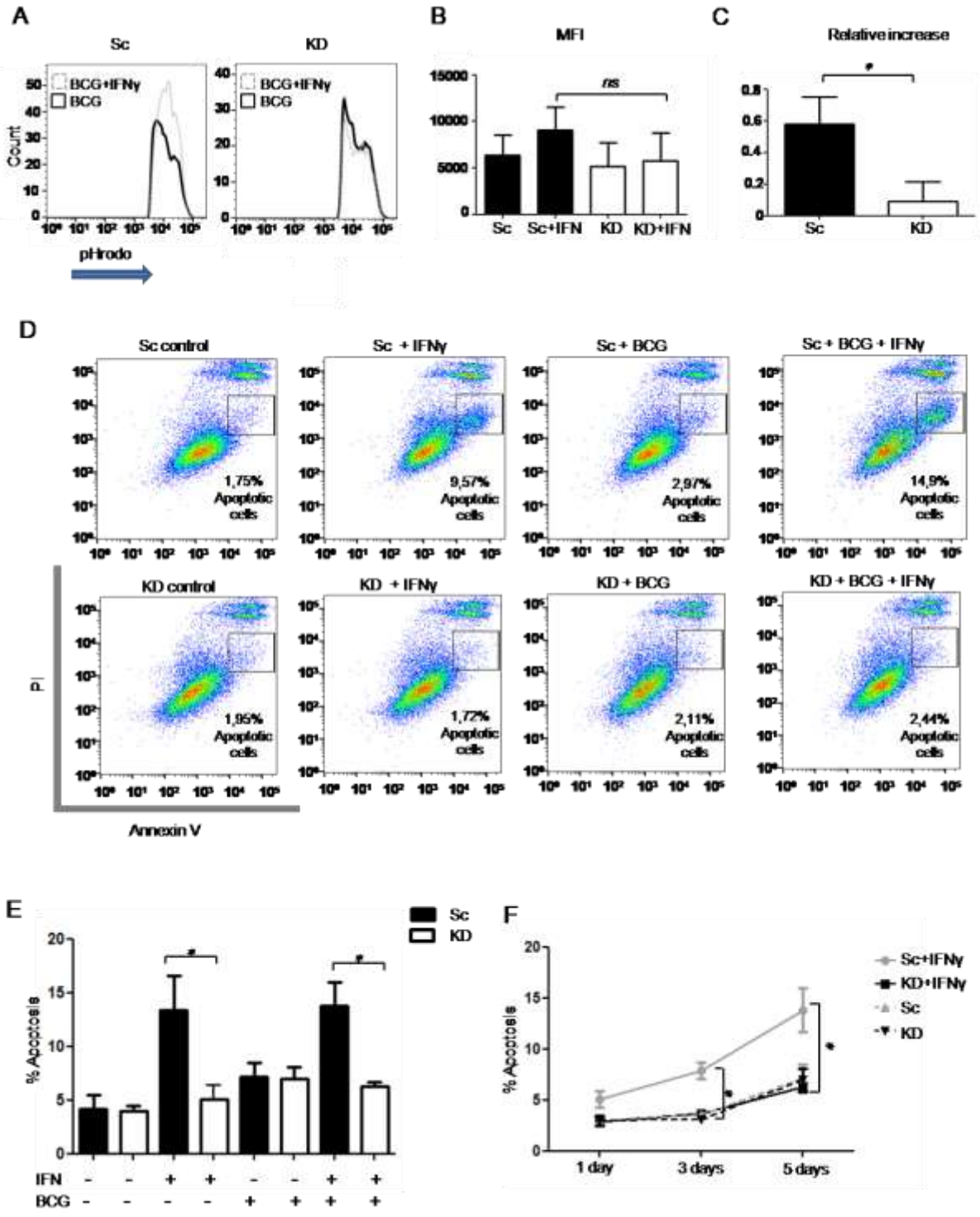


Figure 5. Phagosome acidification and apoptosis is reduced in JAK1-deficient THP1 cells.

(A-C) Flow cytometry measurement of phagosome acidification using detection of pHrodo-labelled BCG 24h post infection of macrophages differentiated from Sc and KD

THP1 cell lines using PMA, with or without prior IFNγ (50ng/ml) stimulation. A displays a representative experiment; B is from six independent experiments. Graphs show mean values ± SE and regulation of the acidification compared to untreated. Two-tailed Mann Whitney test. *P <0.05. Error bars represent the SE. (D-F) Percentage of apoptosis quantified by flow cytometry using annexin V / PI staining in macrophages differentiated from the Sc and KD cell lines at different time points following BCG infection, with or without prior IFNγ (50ng/ml) stimulation. D displays a representative experiment, E and F are from four independent experiments. Two-tailed Mann Whitney test. *P <0.05. Error bars represent the SE.

Chapter II – Role of partial JAK1 deficiency in anti-viral immunity

Variable impact of partial JAK1 deficiency on STAT1 phosphorylation and expression of IFNα-inducible genes in EBV-B cells and fibroblasts

Next, we sought to examine the functional effect of partial JAK1 deficiency on anti-viral response as our patient showed surprisingly mild viral susceptibility given the important role of IFNα for the protective immunity to viruses in humans (74,134,135,202). We studied JAK1-mediated activation of STAT1 proteins in response to IFN stimulation in EBV-immortalized B cells and fibroblasts of the patient with partial JAK1 deficiency and healthy controls, as these lines have been previously utilized for the study of viral susceptibility in patients with PID.

STAT1 phosphorylation was significantly reduced following IFNγ stimulation in skin fibroblasts of the patient with partial JAK1 deficiency but, surprisingly, comparatively preserved in response to IFNα-stimulation (Fig. 6A,B). In keeping with this finding, we observed no difference in mRNA upregulation of the IFNα-inducible gene, *MX1*, following IFNα-stimulation (Fig. 6C). By contrast, EBV-B cells from the patient demonstrated significantly impaired STAT1 phosphorylation in response to IFNα-stimulation with a trend towards less *MX1* mRNA upregulation (Fig. 6D,E). IFNγ-responses were not tested in EBV-B cells as this cell type is resistant to IFNγ-stimulation, requiring extremely high concentrations of IFNγ. Together, our data indicate that impaired IFNα signalling was more profound in EBV-B cells than fibroblasts with partial loss of JAK1 function.

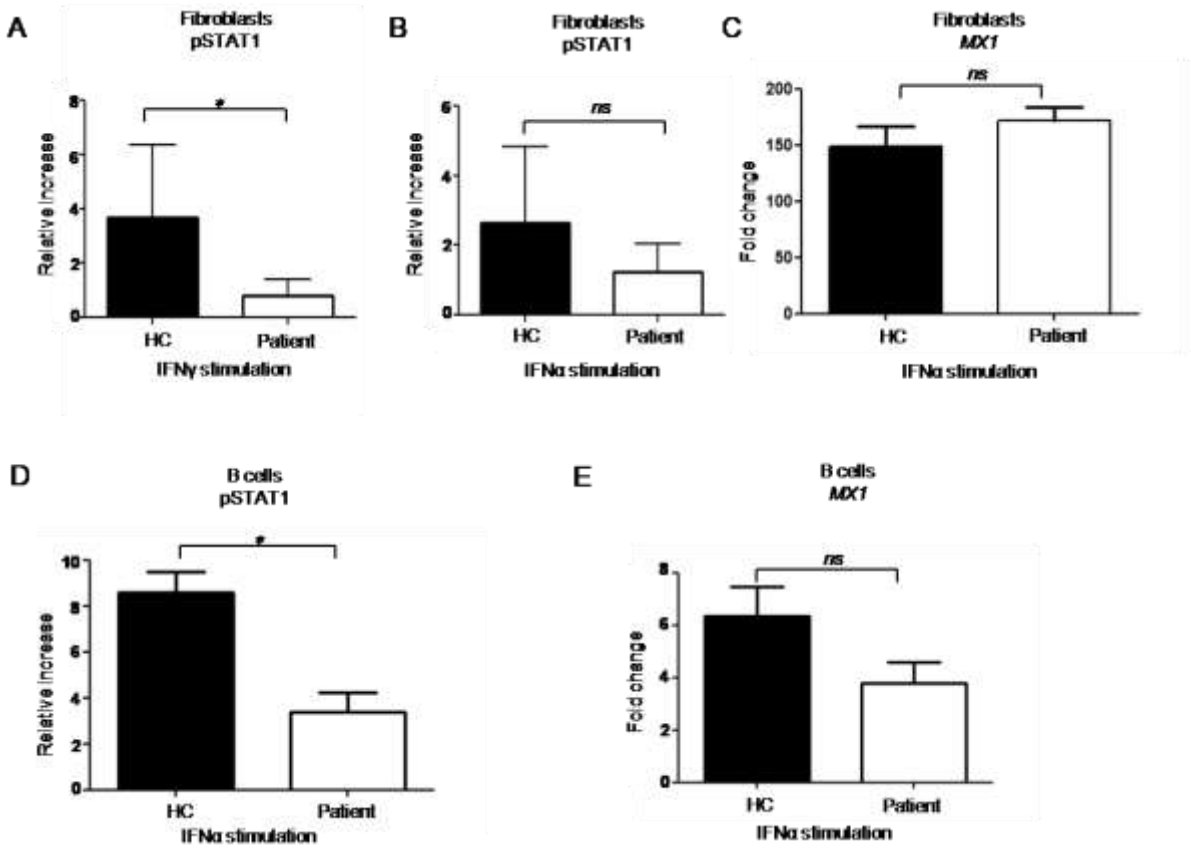


Figure 6. Impaired STAT1 phosphorylation and expression of IFNα inducible genes in EBV-B cells and fibroblasts of the patient with JAK1 deficiency

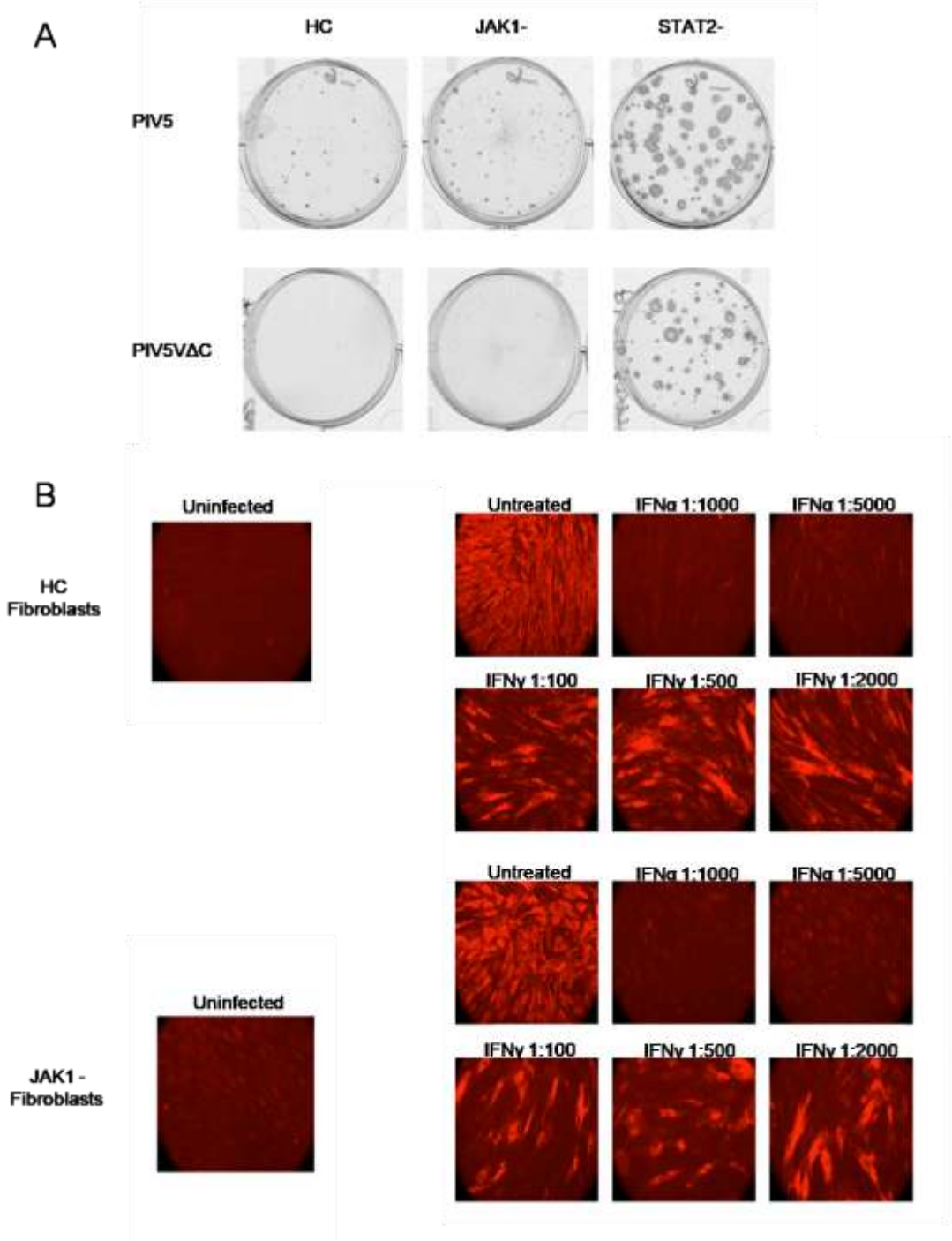
(A,B) Analysis of JAK/STAT signalling by flow cytometry in control and JAK1 deficient fibroblasts after stimulation with IFNγ (100ng/ml) and IFNα (10^3 IU/ml) respectively. (D) Analysis of JAK/STAT signalling by flow cytometry in control and JAK1 deficient EBV-B cells after stimulation with IFNα (10^5 IU/ml). (C,E) RTqPCR analysis of *MX1* expression from control and JAK1 deficient EBV-B cells and fibroblasts after stimulation with IFNα for 24h. (A,B,D) Data are from three independent experiments. (C,E) Data are from four independent experiments. Two-tailed Mann Whitney test. Graphs shows mean values ± SE and regulation of the expression compared to untreated. *P <0.05. Error bars represent the SE.

Partial JAK1 deficiency impairs anti-viral response in EBV-B cells but not in fibroblasts

To test the degree of viral susceptibility in JAK1 deficient cells, we utilised established viral infection models in both fibroblast and EBV B-cells (134,135). We used PIV5 and highly attenuated recombinant strains of PIV5 (PIV5VΔC) that lack defined functional IFN antagonists (33,34). This virus is weakly virulent forming only pinpoint plaques in cells that produce and respond to IFN but with ability to form large plaques if the IFN system is impaired. As previously shown (135), fibroblast monolayers from patients with STAT2 deficiency supported the formation of large plaques (infected cells) of PIV5 and PIV5VΔC, demonstrating uncontrolled viral infection resulting from failure of the type I IFN response (Fig. 7A). Fibroblast monolayers from healthy control and the patient with partial JAK1 deficiency prevented large viral plaque formation indicating successful viral control (Fig. 7A).

We also tested whether partial JAK1 deficiency altered the capacity of fibroblasts to respond to exogenous addition of IFNα and IFNγ treatment to control PIV5 infection. Using immunostaining to visualize intracellular virus, loss of viral fluorescence was seen during successful suppression of viral infection in healthy control fibroblasts treated with IFNα (Fig. 7B). Comparable viral suppression was mediated by patient fibroblasts after IFNα stimulation supporting our observation of preserved IFNα responses in fibroblasts with reduced JAK1-fucntion. Similarly, a lower degree of viral suppression seen after IFNγ stimulation in healthy control fibroblasts was not impacted by partial JAK1 deficiency over a range of IFNγ doses (Fig. 7B).

To further test the impact of partial JAK1 deficiency on host viral protection we used a separate model in which EBV-B cells are infected with VSV. As expected, healthy control EBV-B cells controlled viral infection when treated with exogenous IFNα, evidenced by lower viral titers compared with untreated cells at 24h and 48h after infection (Fig. 7C). In contrast, EBV B-cells from the patient with partial JAK1 deficiency exhibited no reduction in viral titers in the presence of IFNα indicating a significantly reduced response to IFNα. In this assay VSV titers in infected patient EBV-B cells were comparable to STAT1-deficient EBV B-cells after 24h and 48h of infection (Fig. 7C). Together these results suggest that partial JAK1 deficiency results in impaired viral protection with variable impact according to the cell type involved.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HC
JAK1-
STAT2-
PIV5
PIV5VΔC
B
Uninfected
Untreated
IFNα 1:1000
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HC Fibroblasts
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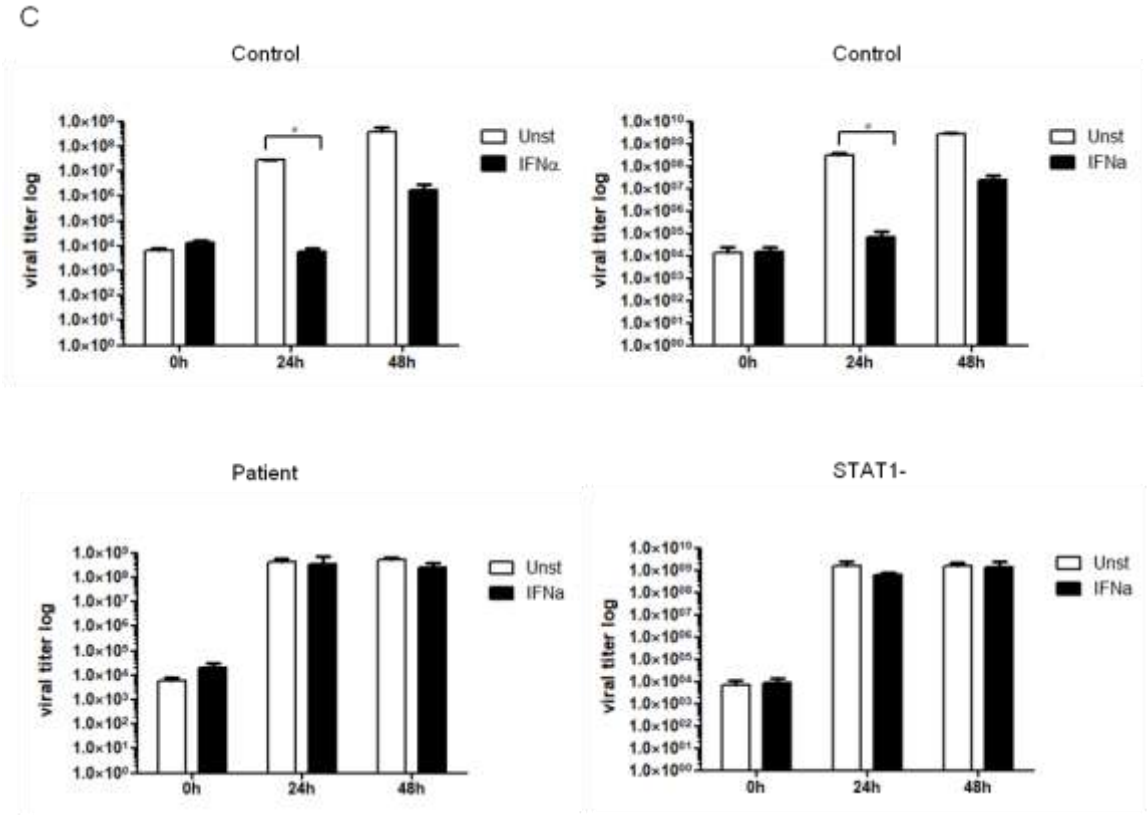

Figure 7. Impaired in vitro antiviral response in EBV-B cells but not fibroblasts of the patient with JAK1 deficiency

(A) Relative plaque sizes of PIV5 and the IFN-sensitive PIV5VΔC virus visualised by immunostaining in fibroblasts from patients with JAK1 deficiency, STAT2 deficiency and healthy control 5 days post infection. (B) Visualisation of PIV5 virus-infected cells by immunofluorescence in control and JAK1 deficient patient fibroblast monolayers, 48 hours post infection with or without IFNα (105 IU/ml) pre-treatment. (C) Determination of VSV viral load (expressed as log10TCID50/ml) at 24 and 48 h in EBV–B cells from the patient with JAK1 deficiency, a patient with complete STAT1 deficiency, and two healthy controls (C1 and C2), with or without pre-treatment with 105 IU/ml IFNα. Data A and B display a representative experiment, C is from three independent experiments. Significance bars are shown when $p < 0.05$ (the experiments related to data C were performed by Michael Ciancanelli in St. Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller University, NY, USA)

Chapter III – Role of partial JAK1 deficiency in cancer susceptibility

Partial JAK1 deficiency impairs STAT1 phosphorylation and expression of IFNγ-inducible genes in hTERT urothelial cells

We used a JAK1-deficient cell model to examine JAK1 function in hTERT-immortalized urothelial cells. We generated a JAK1 KD cell line using lentiviral vectors expressing shRNA sequences. Compared to Sc shRNA, JAK1 shRNA substantially reduced *JAK1* mRNA expression (Fig. 8A). To confirm functional knock down of JAK1, we studied JAK1-mediated activation of STAT1 proteins in response to IFNγ stimulation, using flow cytometry. We observed a significant decrease in STAT1 phosphorylation following IFNγ stimulation in the KD cell line compared to non-transduced and Sc shRNA lines ($p<0.05$) (Fig. 8B,C). IRF1 mRNA expression, was also significantly lower in KD than Sc lines following stimulation with IFNγ, indicating impaired downstream gene regulation in JAK1 deficiency (Fig. 8D). Reduced responses to IFNγ were not due to alteration in expression of the IFNγR as Sc and KD hTERT urothelial cells displayed comparable IFNγR surface expression both at baseline and following IFNγ stimulation (Fig. 8E).

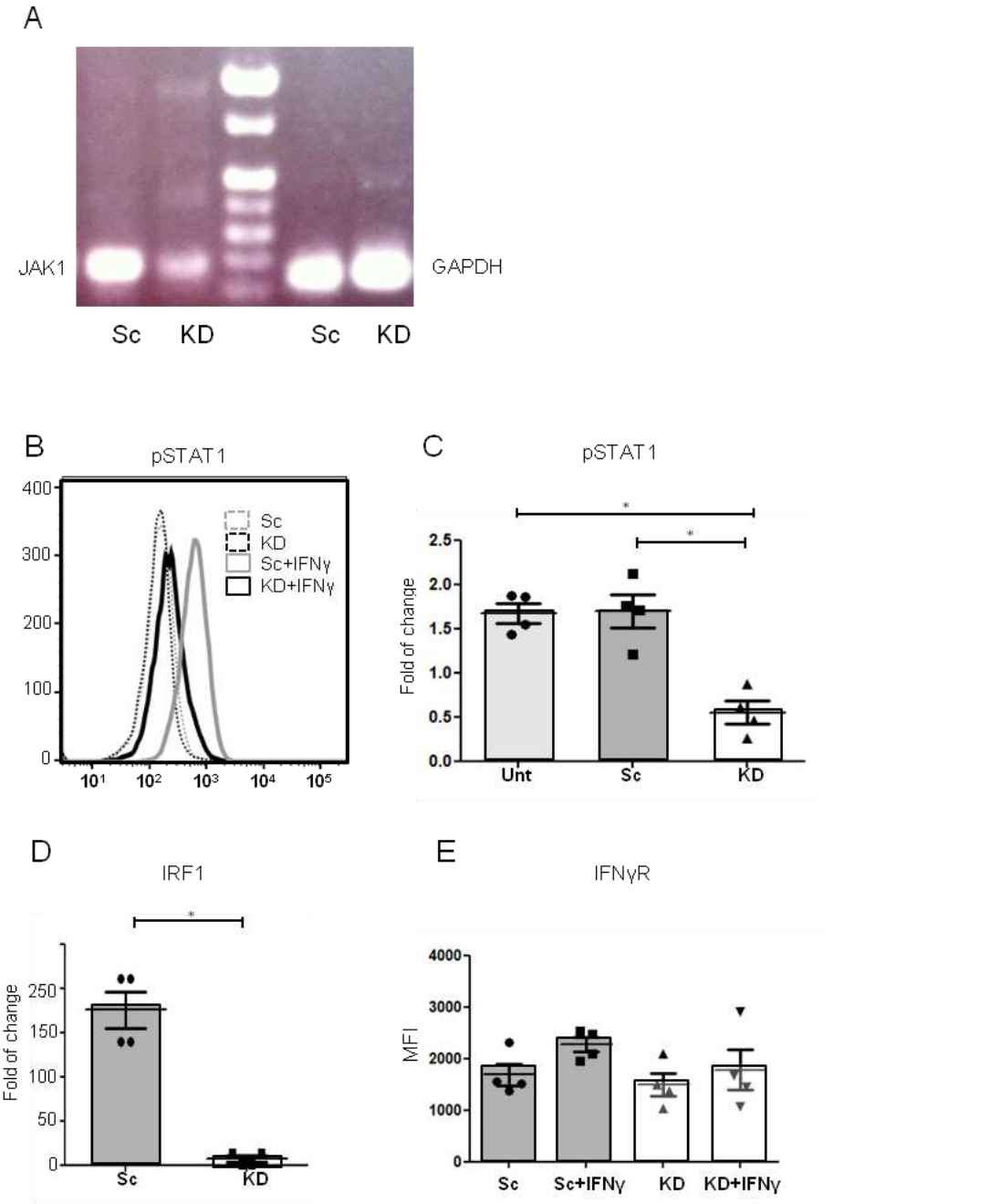

Figure 8. STAT1 phosphorylation and expression of IRF1 is impaired in JAK1-deficient hTERT urothelial cells.

(A) hTERT urothelial cells were transfected with vectors expressing JAK1 shRNA and scrambled control. RT-PCR analysis of JAK1 expression. Data representative from two independent experiments. (B,C) Analysis of JAK/STAT signalling by flow cytometry in untransduced (Unt), Sc and KD hTERT urothelial cells after stimulation with IFNγ (1ng/ml) for 24h. Data B is from a representative experiment, data C is from three independent experiments. Two-tailed Mann Whitney test. (D) RTqPCR analysis of *IRF1* expression from KD and Sc hTERT urothelial cells after stimulation with IFNγ

(1ng/ml). Data is from four independent experiments. Two-tailed Mann Whitney test. *P <0.05 Error bars represent the SE. (E) IFNγR basal expression and after stimulation with IFNγ (5ng/ml) for two days. Data is from three independent experiments. One-way ANOVA with Tukey's Multiple Comparison post-test.*P <0.05. Error bars represent the SE.

JAK1 deficiency alters MHC, ICAM-1 and PD-L1 expression in hTERT urothelial cells

It was previously shown that IFNs regulate expression of MHC, ICAM-1 and PD-L1 in cancer cells (9–11). We examined the expression of these cell surface molecules in JAK1-deficient and Sc hTERT urothelial cells before and after IFNγ treatment (Fig. 9). While basal expression of MHC II was undetectable in both KD and Sc cells, IFNγ induced expression of MHC II in control cells that was significantly lower in the KD cells ($p<0.05$) (Fig. 9A,B). As described for gynecological cancer cells bearing somatic JAK1 mutations (17), we also observed a trend towards lower surface MHC I expression on the cell surface of by JAK1-deficient hTERT urothelial cells compared with control cells both at baseline and after IFNγ stimulation, however, MHC I was upregulated by both control and KD cells although this did not achieve with no statistical significance significant difference (Fig. 9C,D). Expression of ICAM-1 was slightly reduced in KD cells at baseline and expressed at significantly lower levels after IFNγ stimulation compared with Sc control ($p<0,05$. Fig. 9E,F). In keeping with the effects of other reported somatic mutations associated with IFNγ resistance in cancer cells (22,23), we observed reduced PD-L1 expression after IFNγ stimulation in KD cells compared to Sc ($p<0,05$. Fig. 9G,H). Together these data indicate that JAK1 functions regulate the expression of multiple immunomodulatory cell surface molecules in urothelial cells and that JAK1-deficiency alters urothelial cell phenotype.

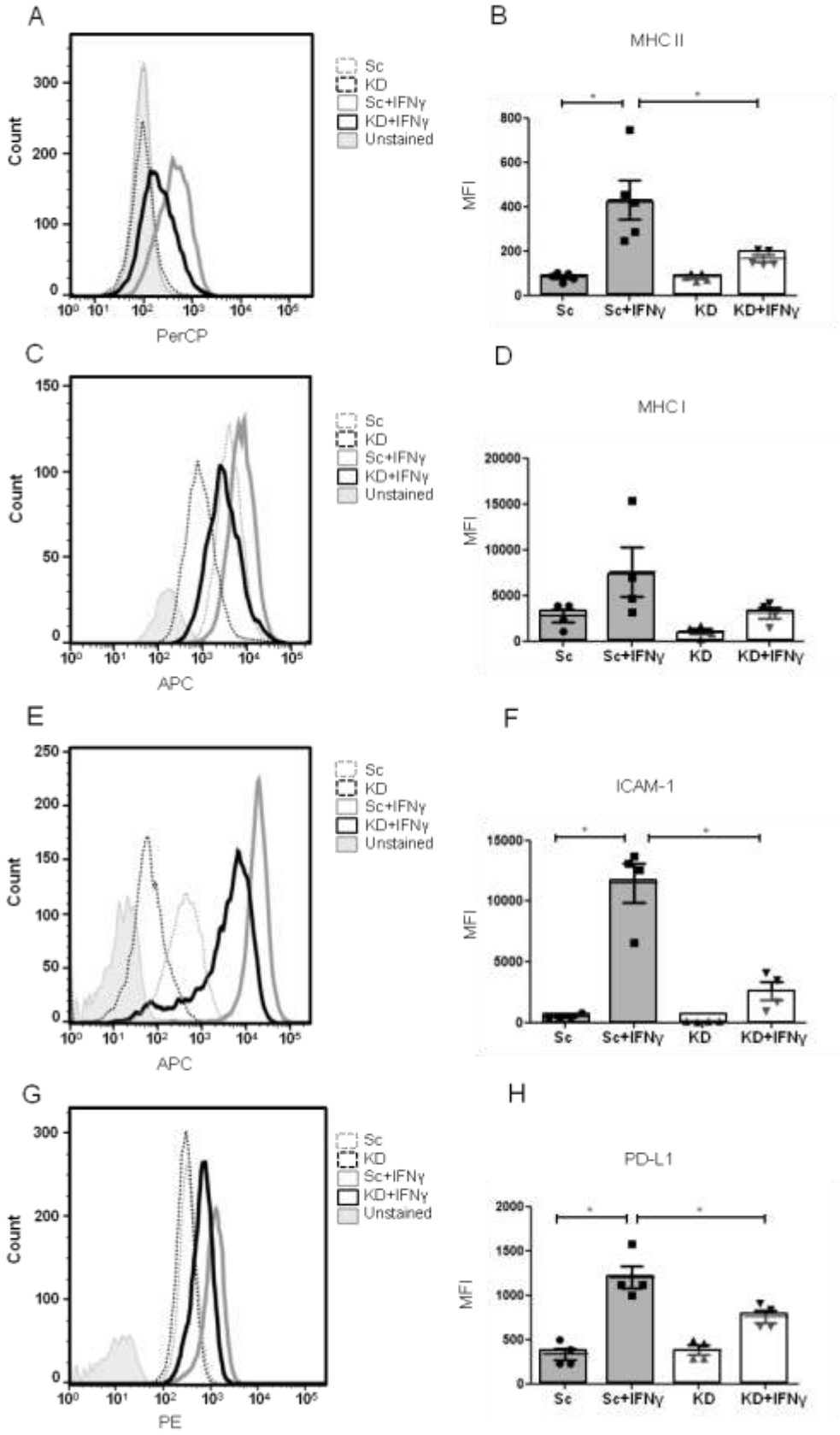
A
Count
PerCP
Sc
KD
Sc+IFNγ
KD+IFNγ
Unstained
B
MHC II
MFI
Sc
Sc+IFNγ
KD
KD+IFNγ
C
Count
APC
D
MHC I
E
F
ICAM-1
G
PE
H
PD-L1

Figure 9. Analysis of MHC I/II, ICAM-1 and PD-L1 expression in hTERT urothelial cells by Flow Cytometry.

KD and Sc hTERT urothelial cells were stimulated with IFNγ for two days and IFNγR expression was analysed by flow cytometry. Data is from three independent experiments. (A,B) MHCII expression in KD and Sc hTERT urothelial cells after stimulation with IFNγ (5ng/ml) for three days. Data is from four independent experiments. (C,D) MHC I and (E,F) ICAM-1 basal expression and in response to IFNγ (5ng/ml) after 24h. Data are from four independent experiments. (G,H) PD-L1 basal expression and after stimulation with IFNγ (5ng/ml) for two days. Data is from three independent experiments. One-way ANOVA with Tukey's Multiple Comparison post-test. Graphs show mean values ± SE and regulation of the expression compared to untreated.*P <0.05. Error bars represent the SE.

JAK1 deficiency impairs apoptosis in response to IFNγ in hTERT urothelial cells

JAK1 KD had no effect on basal growth kinetics of immortalized hTERT cells assessed using an Alamar Blue (AB) reduction assay. In both Sc and KD cell lines, the culture biomass increased steadily over 4 days, indicating a similar increase in cell number over time. IFNγ stimulation significantly inhibited cell growth in the control cell line (Fig. 10A; $p < 0.05$), in keeping with an anti-proliferative effect and/or increased cell death. By contrast, growth kinetics of the JAK1-deficient hTERT urothelial cell population showed no significant change following IFNγ treatment (Fig. 10A). To further investigate this, we tested whether JAK1-deficient cells are resistant to IFNγ-induced apoptosis. IFNγ induced apoptosis in Sc hTERT urothelial cells which was significantly lower in the JAK1-deficient cell line ($p < 0.05$) (Fig. 10B,C), indicating that JAK1 is required for IFNγ-mediated apoptosis in urothelial cells. Together, our data support a requirement for JAK1 in regulating the urothelial cell response to IFNγ.

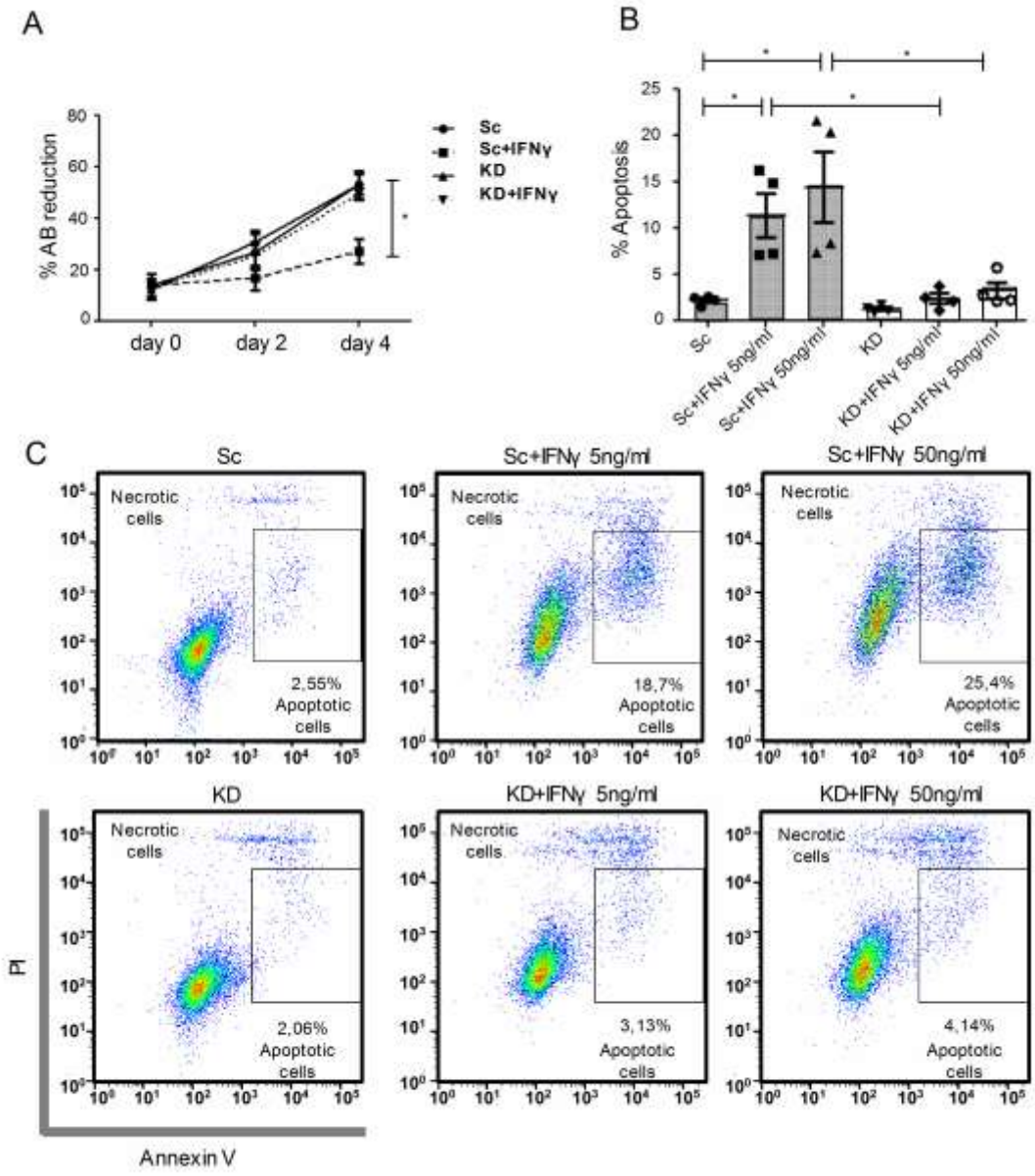

Figure 10. JAK1-deficient hTERT urothelial cells demonstrate preserved population growth and reduced apoptosis in response to IFNγ.

(A) Cells were cultured with Alamar Blue (AB) dye and stimulated with IFNγ (5ng/ml) for the given time points. The capacity of viable cells to reduce AB dye was used as a proxy for cell number. Data A is from five independent experiments. (B,C) KD and Sc hTERT urothelial cells were stimulated with different concentrations of IFNγ for 5 days. Percentage of apoptosis was quantified with Annexin V/PI apoptosis detection kit by flow cytometry. Data B is from five independent experiments, data C is from a representative experiment. One-way ANOVA with Tukey's Multiple Comparison post-test. Error bars represent the SE.

Defective lymphocyte-mediated killing of JAK1-deficient hTERT urothelial cells after IFNγ stimulation

We next sought to establish whether the reduced MHC class I and ICAM-1 levels observed would impair immune cell recognition of JAK1-deficient hTERT urothelial cells (205–208). We tested this using a lymphocyte assay where killing can be mediated by NK or CD8+ T cells through shared a perforin-granzyme and death receptor/death ligand mechanisms. IFNγ pre-treatment of the Sc hTERT cell line resulted in significant enhancement in urothelial cell lysis by third party, IL-2 expanded primary lymphocytes (measured as necrotic cells, $p < 0.05$. Fig. 11A,B). Importantly, lymphocyte mediated cell death was significantly reduced in KD compared to Sc cells following IFNγ pre-treatment ($p < 0.05$. Fig. 11C), suggesting that JAK1 deficiency confers resistance to immune cell killing.

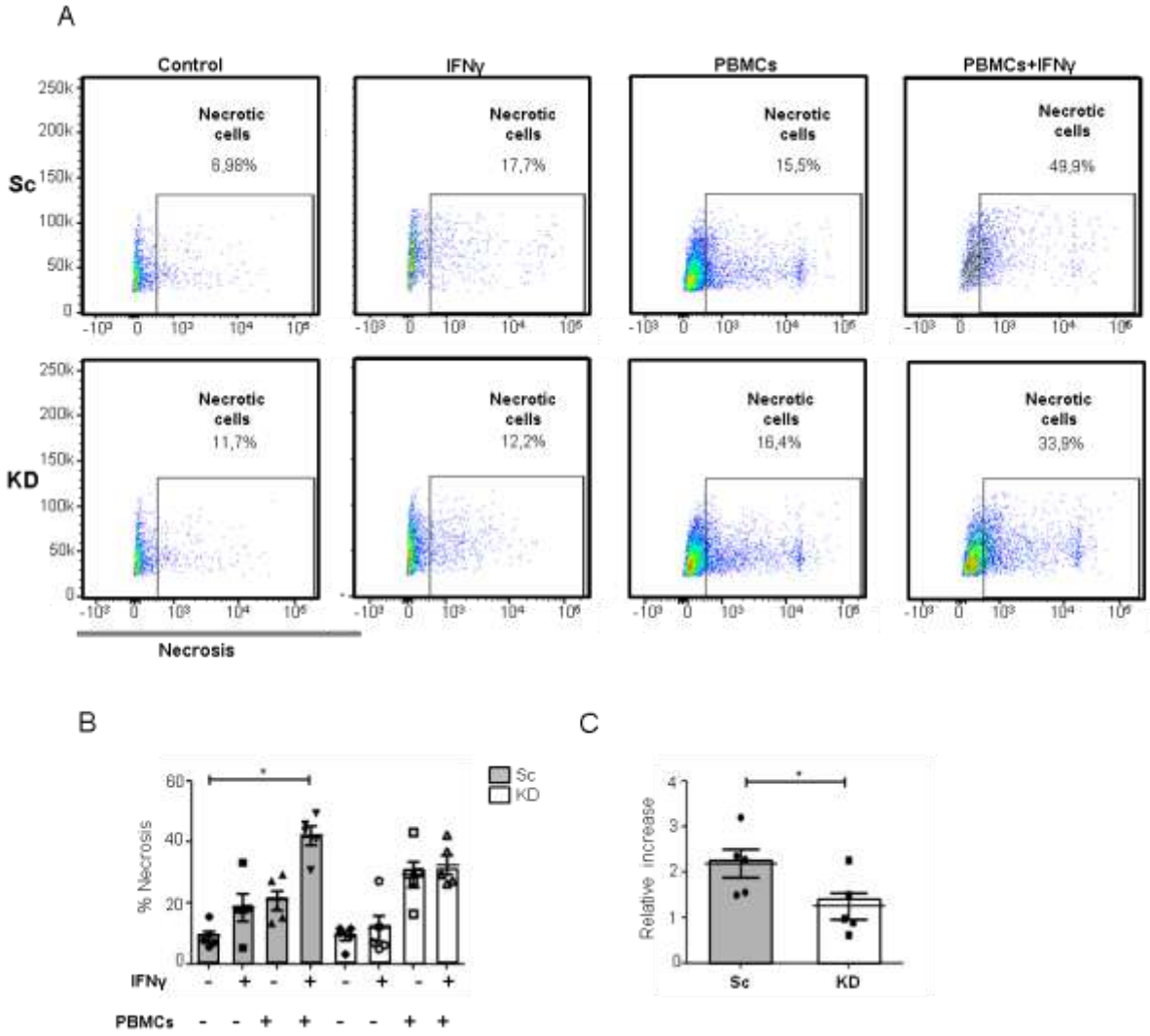

Figure 11. JAK1-deficient hTERT urothelial cells showed reduced lymphocyte-mediated killing in response to IFNγ.

JAK1-deficient and Sc hTERT urothelial cells were pretreated or not with IFNγ and kept in co-culture overnight with IL-2 + monocyte-depleted PBMCs. (A,B) Necrosis induction in urothelial cells. One-way ANOVA with Dunn's multiple comparisons post-test. (C) Relative change of necrosis compared to untreated. Two-tailed Mann Whitney U test. Data is from five independent experiments. *P < 0.05. Error bars represent the SE.

Role of JAK1 in urothelial cytodifferentiation

Histological examination of the invasive urothelial carcinoma found in the JAK1-deficient patient revealed a suppressed urothelial differentiation phenotype. Given the dual role of IRF1 in both IFNγ signaling and PPARγ-mediated urothelial differentiation, this led us to test experimentally whether JAK1 has a role in urothelial cell differentiation.

NHU cell cultures were induced to differentiate by coactivation of PPARγ and inhibition of EGFR signalling, using a combination of troglitazone and PD153035 (TZ/PD) (191). These conditions induce gene expression changes associated with urothelial differentiation via the PPARγ-dependent induction of intermediary transcription factors, including FOXA1 and IRF1 (188). As *IRF1 knock down* limits uroplakin expression (188) and *IRF1* induction was impaired in JAK1-deficient cells (Fig. 2b), we tested by RT-qPCR whether IFNγ modulates expression of genes associated with IFNγ signalling and urothelial cytodifferentiation pathways. As expected, TZ/PD induced up-regulation of *PPARG* and *FOXA1* transcripts (Fig. 12A,B). IFNγ alone had a small effect resulting in a weak up-regulation of *IRF1*, *PPARG* and *CIITA* (Fig. 12A,C,D). Surprisingly however, stimulation with IFNγ+TZ/PD up-regulated all four genes and substantially increased expression of *IRF1*, *FOXA1* and *CIITA* compared with TZ/PD alone (Fig. 12A-D). These data suggest that IFNγ not only induces MHC II expression but significantly enhances PPARγ-mediated differentiation and supports a previously unknown role for JAK1 in regulating urothelial cytodifferentiation.

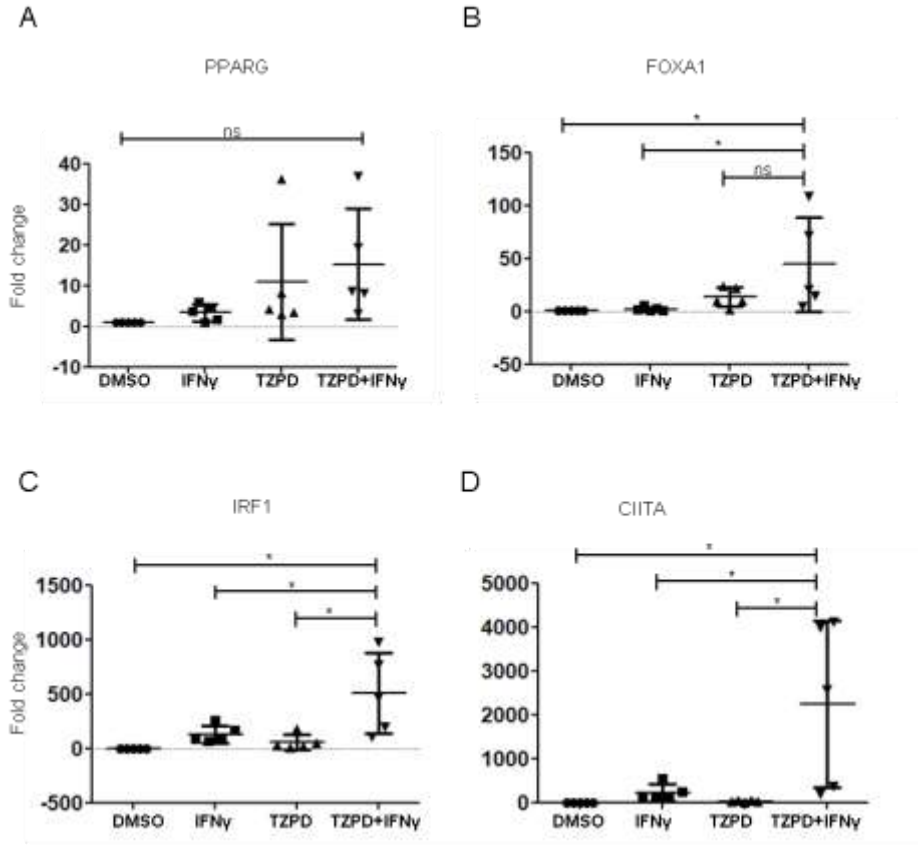

Figure 12. Expression of IFNγ-regulatory genes and intermediary differentiation transcription factors in NHU cells by RT-qPCR.

RT-qPCR analysis of NHU cell gene expression, with or without IFNγ (200U/ml) and/or TZ (1 μM) + PD153035 (1 μM) stimulation for 48h compared with the vehicle-only (0.1% DMSO) control. Data are from five independent experiments derived from five different donors. One-way ANOVA with Tukey's Multiple Comparisons Test. *P<0.05. Error bars represent the SE.

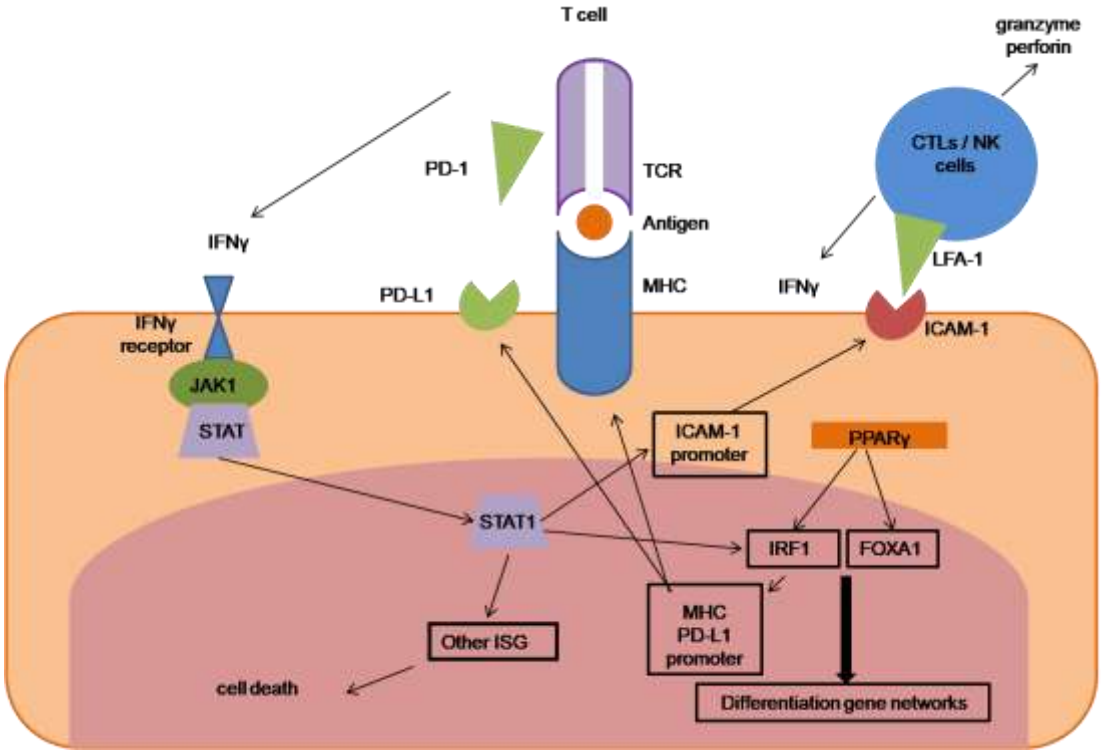

Figure 13. Model for the role of JAK1 in immune recognition and cytodifferentiation of urothelial cells.

JAK1 transduces signals from the IFNγ receptor through phosphorylation of STAT1. In co-operation with the PPARγ pathway, JAK1 signalling activates transcription of IRF1 and FOXA1, regulating urothelial cell differentiation. In addition, JAK1 mediates urothelial cell upregulation of MHC I, MHC II, ICAM-1 and PD-L1 in response to exogenous the IFNγ, promoting immune recognition through T- and NK-cells interaction.

DISCUSSION

The dissection of clinical and immunological phenotypes associated with germline mutations in JAKs and STATs has been essential to define the pathogenesis of the disorders of these genes. However, many of the immunological and clinical phenotypes seen in these patients remain unexplained at the molecular and cellular levels; possibly due to complex regulation and interactions of these signalling pathways.

We recently reported the first case of human germline loss of function hypomorphic mutations in *JAK1*, which resulted in a functional JAK1 partial deficiency with impaired phosphorylation of several STATs, affecting signalling downstream multiple signalling pathways. The disease manifested clinically with syndromic susceptibility to atypical mycobacterial disease and early onset bladder carcinoma. It is likely that impaired phosphorylation of several STAT proteins contributed to the immunodeficiency manifested by the patient impacting multiple cell types.

JAK1 is a ubiquitously expressed, having impact beyond the immune system. The results from analyses of cells and tissues derived from JAK1 KO mice demonstrated that there is an absolute requirement for JAK1 in mediating biologic responses to IFNα and IFNγ in certain cell types, but also it plays a role in mediating intracellular signalling from multiple other cytokine receptors (5). JAK1-deficient mice failed to nurse and died perinatally, possibly as a result of neurological defects. These mice also had reduced numbers of thymocytes, pre-B cells and mature T and B lymphocytes, suggesting a role for JAK1 in immune cell development although the impact of JAK1

deficiency on immune cell function was not tested. No humans have been described with complete loss of JAK1 function, which may reflect an impact on development and function of essential non-immune tissues. Our patient presented with developmental delay and recurrent mycobacterial infections, showing a less severe presentation that the mouse model, presumably because of the partial nature of the JAK1 deficiency (5).

Impact of partial JAK1 deficiency on mycobacterial susceptibility

IFNγ-activated macrophages are more resistant to mycobacterial infection by the induction of several discreet mechanisms that promote mycobacterial killing (209–212), such as phagosome maturation and apoptosis (102,114,200,213,214). An increasing number of rare disease-causing mutations are described to cause isolated or syndromic MSMD (119,121,215). Either directly or indirectly, all impair the production of or the response to IFNγ, indicating that IFNγ mediated immunity is essential to control intramacrophagic infections.

We investigated the specific roles of JAK1 in myeloid cells during mycobacterial infection using a THP1 cell line with partial JAK1 deficiency generated using lentiviral vectors expressing shRNA sequences to mimic the hypomorphic JAK1-deficiency seen in our previously described patient (2). Using in vitro infection models with BCG, JAK1-deficient THP1 cells supported enhanced mycobacterial survival after IFNγ stimulation reminiscent of uncontrolled mycobacterial replication previously reported in IFNγR1-deficient human iPSC-Derived Macrophages (216). We observed similar findings using *Salmonella typhimurium*, another intracellular pathogen known to require

IFNγ signalling for control of the bacterial replication, suggesting that JAK1 defects may be important beyond mycobacterial infections.

We identified a number of specific IFNγ-related functions that were impaired in THP1 cells with partial JAK1-deficiency that are known to impact mycobacterial protection. Firstly, JAK1-deficient THP1 cells showed impaired IFNγ-mediated upregulation of *CIITA*, essential for transcriptional activity of the MHC II promoter, and *IRF1*, one of the major primary response genes induced by STAT1 signalling involved in the transcription of a large number of secondary IFN response genes (89). These results show impaired downstream gene transcription in partial JAK1 deficiency. Among the mechanisms promoting the control of mycobacterial infection in myeloid cells (102,103,114,199–201), we observed reduced phagosome acidification capacity and apoptosis in the JAK1 deficient cell line after IFNγ stimulation. Intracellular survival of pathogenic mycobacteria is dependent on inhibition of maturation of the phagosomes containing these pathogens into functional phagolysosomes. Mycobacteria can escape the macrophages' bactericidal effectors by interfering with phagosome-lysosome fusion. IFNγ activation renders the macrophages capable of killing intracellular mycobacteria by overcoming the phagosome maturation block, exposure to microbicidal effectors, modulation of presentation of microbial antigens and induction of apoptotic cell death; mechanisms that could be affected in defects of the IFNγ pathway, leading to the absence of control of mycobacterial infection (33,114,116,217).

Therefore, we concluded that in myeloid cells, JAK1 is non-redundant for multiple aspects of the IFNγ-response required to control intracellular bacterial infection. Our findings suggest that impaired myeloid cell function was a key contributing factor to mycobacterial infection in our JAK1-deficient patient. Our data shows that partial disruption of JAK1 signaling is sufficient to impair anti-mycobacterial protection, which has implications for the increasing use of JAK1 inhibitors in other areas of medicine. It is intriguing that partial defects of three genes involved in the response to IFNγ (*IFNGR1, STAT1* and *JAK1*) underlie the pathogenesis of osteomyelitis associated to mycobacterial disease (2,101,121). Despite susceptibility to weakly virulent mycobacteria, our patient did not succumb to infection but recovered through the use of prolonged antibiotic treatment and long-term antibiotic prophylaxis, suggesting partial preservation of host defense. Further work is required to determine what level of residual JAK1 is required to preserve immune competence.

Impact of partial JAK1 deficiency on viral susceptibility

Unusual viral infections are often a sign or complication of PID. This has been seen in T cell defects as well as defects of the myeloid compartment. Nearly all T cell disorders can be associated with increased susceptibility to warts; however, there is a small group of PIDs that have warts as a cardinal feature, presenting severe and recurrent warts. Chronic papillomavirus are particularly characteristic of PID and may predispose to cutaneous carcinoma (218). There are more than 200 strains of human papillomavirus (HPV) and the diverse strains have variable malignant potential and tissue tropism. Our patient presented with planar warts of the forehead that were chronic and resistant to imiquimod treatment. He did not have other clinically apparent HPV infection or other

severe viral infections. This was somewhat surprising, given the known role of JAK1 in signaling from IFNα/βR.

Therefore, we set out to examine the ability of skin fibroblasts and EBV-B cells from our JAK1-deficient patient to develop antiviral responses in vitro. Surprisingly, we observed little reduction of IFNα-induced STAT1 phosphorylation in JAK1-deficient fibroblasts and no detectable susceptibility to viral infection, suggesting that residual JAK1 activity was sufficient to preserve the IFNα response for the control of viral proliferation in that cell type. These results were unexpected given the previous work done in fibroblasts of this patient which demonstrated a moderate reduction in IFNα-induced pSTAT1 by western blotting and significant reduction in IFNα related genes at similar doses of IFNα (2) (Appendix). This finding led us to sequence the cells which confirmed presence of the expected mutations. The discrepancies may be explained by one of a number of possibilities. Firstly, the activity of IFNα in commercially available preparations differs which could reduce direct comparability between the two sets of experiments. Secondly, different techniques were used to determine pSTAT1 levels; flow cytometry was utilised here which may have a different sensitivity to detect moderate changes in pSTAT1. Thirdly, passage number can impact cell line function and fibroblasts of a higher passage number were utilised here compared with the earlier study. Finally, *MX1* was measured here as an interferon-responsive gene whereas a different set of downstream genes was measured in the Eletto study. The impact of the degree of reduced pSTAT1 demonstrated in the Eletto study on viral protection in vitro was not tested. Here we found that the relative preservation of STAT1 phosphorylation and signalling in the patient fibroblasts preserved functional antiviral immunity of these

cells mediated by IFNα in vitro. In contrast, EBV B-cells from our patient had a significantly lower degree of STAT1 phosphorylation compared with control EBV B-cells. Consistent with a greater reduction in IFNα signaling, the patient's EBV-B cells with JAK1 deficiency showed lack of viral protection, suggesting a more pronounced failure of the type I IFN response in hematologic cells. Our findings are in keeping with the previously reported uncontrolled viral replication in EBV-B cells from patients with AR TYK2 or AR STAT1 deficiency that present with both mycobacterial and viral infections (18,121,134). The unusual but not life-threatening viral infections seen in our patient remains surprising, such as the mild phenotype of human STAT2 deficiency (135), suggesting that possibly other mechanisms are involved in anti-viral protection in these disorders. The description of additional patients may broaden the phenotype of JAK1 deficiency in humans and provide opportunities to further assess the relative importance of JAK1 for viral protection in hematopoietic and non-hematopoietic cell types.

Role for partial JAK1 deficiency in malignancy

Many PIDs has been associated with elevated risk of cancer and the number of PID-associated malignancies has increased during the last years together with the improvement of patient survival, due to the development of better treatments and management of infectious complications. Defective immunosurveillance mechanisms and infection with oncogenic viruses seem to have significant contributory roles; and it has been reported that around 20% of carcinomas in patients with PID are associated to infection (219).

JAK1 deficiency could be associated with viral susceptibility that could provide a trigger for neoplastic change in infected cell types. It is already known that some cases of bladder carcinoma may be associated with HPV and Polyomavirus infection (220–222) demonstrating proof of principle that failure to eradicate infection can lead to malignancy in urothelial cells. The presence of HPV and EBV was excluded in the biopsy of the bladder of the patient with partial JAK1 deficiency. However, we can not fully exclude the presence of other viruses that were not specifically investigated, such as polyomavirus. Polyomavirus BK (BKV) infects around 70-100% of the human population, mainly in the urogenital tract, but it has not been associated with disease in immunocompetent individuals. However, in immunocompromised renal transplant recipients, BKV frequently reactivates and may cause nephropathy and ureteral stenosis. It has been reported one case of active BKV replication associated to metastasized bladder carcinoma, adding evidence for the possible implication of BKV in the pathogenesis of bladder cancer in the context of immunodeficiency (223).

Some cancers in patients with PID are not known to be related to infectious agents, suggesting more complex interactions between factors such as genetically altered tumour suppression genes, impaired immunosurveillance and chronic inflammation. Combinations of these factors are thought to serve to increase the risk of malignancy and resistance to treatment in patients with PID (224,225). Upon tumour antigen recognition, T cells produce IFNγ, which through the IFNγR, JAK1/JAK2 and the STAT proteins, resulting the expression of a large number of IFN-stimulated genes with

beneficial antitumour effects, such as increased antigen presentation, direct tumour growth arrest and apoptosis (178).

To date, urothelial carcinoma has not been described as a feature of JAK-STAT related PID although other tumours have been reported in patients with other defects in the IL-12/IFNγ pathway; including disseminated cutaneous squamous cell carcinoma (122,123,125,126). Interestingly, somatic mutations in *JAK1* are seen in high-risk bladder cancer and gynaecological carcinomas (176,177), supporting the idea that defective JAK1 signalling could play a role in the pathogenesis of some epithelial cancers. The early-onset and aggressive nature of the malignancy, along with the fact that *JAK1* is a hotspot for damaging somatic mutations in bladder carcinoma (226), led us to investigate whether impaired JAK1 function could impact intrinsic urothelial cell function and be a specific predisposing factor for urothelial carcinoma. In order to test this hypothesis, we generated a cell model with partial JAK1-deficiency, generated using lentiviral vectors expressing shRNA sequences to mimic the hypomorphic JAK1-deficiency seen in our previously described patient (2).

IFNγ can target tumour cells directly, regulating the expression of genes involve in the inhibition of proliferation and apoptosis induction (75,147,161,163). We show that loss of JAK1 function impaired induction of apoptosis in response to IFNγ suggesting a role for JAK1 in regulating intrinsic urothelial cell homeostasis. In addition, IFNγ can upregulate MHC class I and ICAM-1, among other co-stimulatory molecules. JAK1-deficient urothelial cells demonstrated reduced surface expression levels of ICAM-1 and to a lesser extent MHC class I following IFNγ stimulation, which was associated with

resistance to lymphocyte-mediated cell lysis that is known to depend on cell surface expression of these molecules (205–208).

T cells and NK cells control tumour cells through releasing cytotoxic granules and proinflammatory cytokines, and have been shown to play a key role in the control of metastatic dissemination (227). IFNγ enhances NK cell cytotoxicity through up-regulation of ICAM-1 expression in target cells, promoting their conjugate formation with NK cells (206). Defects in the IFNγ signalling may significantly impair NK cell lysis of target cells and in keeping with this, JAK inhibition has been reported to enhances metastasis in breast cancer models by decreasing NK-cell tumour immunosurveillance (228). Recently it has been described that ICAM-1 also enhanced the susceptibility of tumour cells to antigen-specific lysis by cytotoxic T-lymphocytes (CTLs) (229), and reduced expression of MHC class I molecules by cancer cells represents a potential strategy to escape immune recognition, inducing resistance to T cell-mediated immune detection (230). Thus, impaired MHC class I expression could be involved in reduced tumour cell killing by CTLs. In addition, loss of JAK1 was shown to reduce JAK/STAT1 signalling in tumour cells resulting in loss of tumour immunogenicity in mice. Both type I and II IFN pathways were impaired in the absence of JAK1 in tumour cells, resulting in suppression of anti-tumour T-cell responses by impairing antigen presentation (231). JAK1 is also required for expression of MHC class II that mediates tumour and self-antigen presentation in non-professional antigen presenting cells (232), and it was shown to be significantly reduced in JAK1-deficient urothelial cells after IFNγ stimulation. Together these data suggest that JAK-deficient

urothelial cells are less susceptible to IFNγ-mediated apoptosis, immune cell recognition and immune-mediated cell death.

In addition to impacting immune-related functions, we observed a potential role for JAK1 and IFNγ signalling in urothelial cell differentiation. Urothelium is the transitional epithelium that lines the luminal surface of the bladder and urinary tract, where it acts as a urinary barrier. The urothelium is comprised of basal, intermediate and superficial cell zones, which may be distinguished on the basis of differential cytokeratin (CK) and claudin isotype expression profiles and by the expression of urothelium-specific uroplakins (UPK) by the superficial cells. However, very little is known of the signalling mechanisms that drive the process of urothelial cytodifferentiation (188,233–235).

The nuclear receptor PPARγ is highly expressed in different tissues including the developing and mature urothelium (187), and is implicated in the induction of differentiation of NHU cells. It has been shown that in EGFR-inhibited NHU cell cultures, the activation of PPARγ results in the induction of a differentiation programme, leading to de novo expression of late/terminal differentiation markers, including UPK1a, UPK2, UPK3a, CK20 and claudin (188,192,234).

It has been shown that NHU cells can be induced to differentiate using PPARγ ligands and concurrent EGFR inhibition, for example using TZ/PD (192). In this study, we show that IFNγ had a significant effect on the induction of the transcription factors IRF1

and FOXA1, both known to be involved in urothelial cell differentiation induced by PPARγ activation (188). In particular, IRF1 is a common downstream mediator for PPARγ and IFNγ signalling pathways influencing both urothelial differentiated phenotype and immune cell interactions. This suggests that IRF1 may be a potential novel target for modulating immunotherapy outcomes in urothelial cancer. Further research is needed to understand the mechanisms of IFNγ interaction with PPARγ and EGFR signalling in urothelial cell differentiation and tumorigenesis. These data demonstrate that JAK1 is important for multiple aspects of urothelial cell biology and highlight mechanisms by which loss of JAK1 function may promote tumorigenesis in this cell type.

Relevance for PD-1/PD-L1 blockade therapy

The upregulation of PD-L1 on the tumour cell surface seems to be one of the major ways that tumours appear to avoid immune surveillance (138,236). In order to enhance the immune response to cancer, immune therapies such as vaccination, oncolytic viruses and adoptive cell transfer, seek to induce immune responses to tumour specific antigens and promote a pro-inflammatory environment (138). To overcome the suppressive tumour microenvironment, checkpoint inhibitors have been investigated in order to overcome the immune suppression (237) by blocking the inhibitory pathways of the immune response. Anti-PD-1 and anti-CTLA-4 antibodies have been shown to have good therapeutic effects in many tumours and are currently approved for clinical use. PD-1 blocking immunotherapy has resulted in rates of long lasting anti-tumour activity in patients with metastatic cancers of different histology, including bladder cancer (178,238). We observed that JAK1-deficient urothelial cells demonstrated reduced

surface expression levels of PD-L1 following IFNγ stimulation. These findings are consistent with previously reported data that revealed damaging mutations in the IFNγ signalling pathway associated with metastasis and higher resistance to the checkpoint blocking therapy with anti-PD-L1/PD-1 in a number of tumour types, including bladder cancer (178,207).

Despite the success with checkpoint inhibitors, many patients and tumour types have failed to show clinical responses. Recent reports suggest that loss of IFNγ pathway genes is associated with resistance to anti-PD-1 therapy. LOF somatic mutations in *JAK1/2* were associated to resistance to anti-PD-1 therapy in human melanoma cell lines, through the lack of reactive PD-L1 expression and response to IFNγ (178). Acquired resistance to PD-1 blockade immunotherapy has also been described in patients with melanoma, associated with defects in the pathways involved in IFNγR signalling and in antigen presentation, including *JAK1* and *JAK2* truncating mutations that resulted in a lack of response to IFNγ, including insensitivity to its antiproliferative effects on cancer cells (179). Thus, primary and acquired resistance becomes one of the major obstacles, limiting the effects of PD-1/PD-L1 blockade therapy (178–180). Our data specifically implicate loss of function *JAK1* mutations as a risk factor for lower tumour cell PD-L1 expression which could impair responsiveness to anti-PD-1 therapies used for advanced urothelial carcinoma (239).

One important factor to consider is the heterogeneity between cancers. Classification of tumours according to the gene profile and immunogenicity has begun to help to predict

how they might respond to different immune based treatments (138,240,241). Regarding the importance of IFNγ in cancer diagnostics, IFNγ-associated signatures have a predictive value (150,151,211) for the use of PD-1 or CTLA-4 blockade in various types of malignancies (75,243,244). These accumulating evidences highlight the importance to detect the patients' genetic information of tumours and grade of patients' IFNγ-responsiveness, which will help to establish individualized immunotherapy (179).

Relevance for JAK inhibitor treatment

Inhibition of JAK enzymatic activity has recently become a powerful tool for treatment of several immune, hematologic disorders and solid tumours. JAKs inhibitors has been used for the treatment of myeloproliferative neoplasms, rheumatoid arthritis, inflammatory bowel disease, psoriasis, among others disorders associated to aberrant activation of the JAK/STAT signalling, such as the autoinflammatory interferonopathies and STAT1 GOF, where the inhibition of these associated signalling pathway seems to be effective (245–250). The adverse effects of JAK1 inhibitors are largely predictable based on their biological functions as signal transducers for Type I and Type II cytokines. Despite differences in selectivity between JAK inhibitors, a large overlap exists in their safety profiles, including infections and changes in laboratory parameters.

Our findings contrast with the adverse effect profile published with early trails of the selective JAK1 inhibitors, filgotinib and upadacitinib, where viral infections (particularly herpes zoster and BK viremia) and not mycobacterial disease predominate (251–254). Although most infections associated with JAK1 inhibitors did not necessitate treatment discontinuation, severe and opportunistic infections such as

tuberculosis and osteomyelitis were also reported. Overall, the risk of serious infections appears to be similar to that seen with biological agents. Although increase risk of malignancy with JAK1 inhibitors has not been identified to date, long-term follow-up is needed to understand the risk of malignancy associated with these compounds (247,255).

In view of these findings, sensible precautions prior to the prescription of JAK1 inhibitors could be a screening for latent TB as well as vaccination against herpes zoster such as in biological therapies (255). Close monitoring of possible viral infections and the development of malignancy during treatment would also be recommended. Longer experience with pharmacological JAK1 inhibition and identification of additional patients with germline JAK1 deficiency, including perhaps patients with more common and milder forms of JAK1 deficiency, will allow us to better understand the relative importance of JAK1 for specific cytokine pathways governing host protection and malignancy risk in vivo.

Limitations of the study

For most of the experiments performed in this study, partial JAK1 deficiency was modeled using a knock down approach in order to investigate the impact of partial JAK1-deficiency on interferon (IFN) signalling in different cells types. The main reason for this approach was the lack of primary patient cells available, as disease was fatal in our patient and no other patients with germline JAK1 loss of function mutations have been described to date. However, null mutations/knock down models may give

different results in some conditions to mutations that preserve protein expression with hypomorphic function (32). Future work could aim to re-express the patient mutations in KO cells or generate hypomorphic mutations using gene editing approaches (eg CRISPR/Cas9 with a mutated repair template). The description of new patients with hypomorphic mutations in *JAK1* will help to validate the results of the experiments shown here and clarify the clinical spectrum of the disease.

In summary, we provide the first evidence that partial JAK1 deficiency results in mycobacterial susceptibility by reducing multiple aspects of the IFNγ response in myeloid lineage cells. Our data suggest that the predominant effect of partial JAK1 deficiency is on the IFNγ pathway. Although viral susceptibility was also observed in vitro, this varied according to cell type. The discovery of new inborn errors of known and unknown genes will shed light on the pathogenesis of the disorders involving the JAK/STAT pathway.

Our findings also highlight previously unknown roles for JAK1 in urothelial cell immune recognition and differentiation. Our data suggest that loss of JAK1 function through germline or somatic mutation promotes malignant transformation of urothelial cells which are intrinsically less immunogenic. Our results add further weight to arguments for sequencing urothelial cell tumours for clinical trials of immunotherapy agents to test whether the mutational burden of *JAK1* and other IFNγ-related genes represent a biomarker for responsiveness to treatment in bladder cancer, which can more accurately predict the clinical outcome of these patients.

CONCLUSIONS

1. Effect of partial JAK1 deficiency on mycobacterial susceptibility

- Partial JAK1 deficiency impairs STAT1 phosphorylation and expression of IFNγ-inducible genes in myeloid cells.

- Partial loss of JAK1 function promotes mycobacterial and salmonella survival in myeloid cells.

- JAK1 deficient myeloid cells show altered IFNγ-dependent killing mechanisms, including impaired IFNγ-induced phagosome acidification and apoptosis in myeloid cells.

2. Effect of partial JAK1 deficiency in anti-viral protection

- Partial JAK1 deficiency induces variable impact on STAT1 phosphorylation and expression of IFNα-inducible genes in EBV-B cells and fibroblasts

- Partial JAK1 deficiency impairs anti-viral response in EBV-B cells but not in fibroblasts

3. Effect of partial JAK1 deficiency in cancer susceptibility

- Partial JAK1 deficiency impairs immune surveillance mechanisms including altered MHC, ICAM-1 and PD-L1 expression and impaired apoptosis in response to IFNγ urothelial cells

- Partial JAK1 deficiency is associated with defective lymphocyte-mediated killing of JAK1-deficient urothelial cells after IFNγ stimulation.

- JAK1 has a potential role in urothelial cytodifferentiation

These findings suggest that the predominant effect of partial JAK1 deficiency is on the IFNγ pathway resulting in mycobacterial susceptibility. Although viral susceptibility was also observed in vitro, this varied according to cell type. The findings also highlight previously unknown roles for JAK1 in urothelial cell immune recognition and differentiation, providing a platform for further development of novel biomarkers and targeted therapy development for urothelial carcinoma.

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APPENDIX

1. Congress presentations

XVIIth Meeting of the European Society for Immunodeficiencies. Barcelona, 2016.

Parallel session II: autoimmunity, autoinflammation and PID vanishing at new frontiers:

JAK1 deficiency predisposes to mycobacterial infection and cancer.

V. Daza Cajigal, I. Angulo, K. Nowak, D. Evangelopoulos, T. Mchugh, R.E. Randall, S. Nejentsev, A.J. Thrasher, S.O. Burns.

2. List of publications

1: Lee PP, Lobato-Márquez D, Pramanik N, Sirianni A, **Daza-Cajigal V**, Rivers E, Cavazza A, Bouma G, Moulding D, Hultenby K, Westerberg LS, Hollinshead M, Lau YL, Burns SO, Mostowy S, Bajaj-Elliott M, Thrasher AJ. Wiskott-Aldrich syndrome protein regulates autophagy and inflammasome activity in innate immune cells.Nat Commun. 2017 Nov 17;8(1):1576.

2: Dobbs K, Tabellini G, Calzoni E, Patrizi O, Martinez P, Giliani SC, Moratto D, Al-Herz W5, Cancrini C, Cowan M, Bleesing J, Booth C, Buchbinder D, Burns SO, Chatila TA, Chou J, **Daza-Cajigal V**, Ott de Bruin LM, de la Morena M, Di Matteo G, Finocchi A, Geha R, Goyal RK, Hayward A, Holland S, Huang CH, Kanariou MG, King A, Kaplan B, Kleva A, Kuijpers TW, Lee BW, Lougaris V, Massaad M, Meyts I, Morsheimer M, Neven B, Pai SY, Plebani A, Prockop S, Reisli I, Soh JY, Somech R, Torgerson TR31, Kim YJ, Walter JE, Gennery AR, Keles S, Manis JP, Marcenaro E, Moretta A, Parolini S, Notarangelo LD. Natural Killer Cells from Patients with Recombinase-Activating Gene and Non-Homologous End Joining Gene Defects Comprise a Higher Frequency of CD56bright NKG2A+++ Cells, and Yet Display Increased Degranulation and Higher Perforin Content.Front Immunol. 2017 Jul 17;(8):798.

3: Davide Eletto, Siobhan O. Burns, Ivan Angulo, Vincent Plagnol, Kimberly C. Gilmour, Frances Henriquez, James Curtis, Miguel Gaspar, Karolin Nowak, **Vanessa Daza-Cajigal**, Dinakantha Kumararatne, Rainer Doffinger, Adrian J. Thrasher and Sergey Nejentsev. Biallelic JAK1 mutations in immunodeficient patient with mycobacterial infection. Nat Commun. 2016;7: 13992.

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Biallelic JAK1 mutations in immunodeficient patient with mycobacterial infection

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Mutations in genes encoding components of the immune system cause primary immunodeficiencies. Here, we study a patient with recurrent atypical mycobacterial infection and early-onset metastatic bladder carcinoma. Exome sequencing identified two homozygous missense germline mutations, P733L and P832S, in the JAK1 protein that mediates signalling from multiple cytokine receptors. Cells from this patient exhibit reduced JAK1 and STAT phosphorylation following cytokine stimulations, reduced induction of expression of interferon-regulated genes and dysregulated cytokine production; which are indicative of signalling defects in multiple immune response pathways including Interferon-γ production. Reconstitution experiments in the JAK1-deficient cells demonstrate that the impaired JAK1 function is mainly attributable to the effect of the P733L mutation. Further analyses of the mutant protein reveal a phosphorylation-independent role of JAK1 in signal transduction. These findings clarify JAK1 signalling mechanisms and demonstrate a critical function of JAK1 in protection against mycobacterial infection and possibly the immunological surveillance of cancer.

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Primary immunodeficiencies (PIDs) are genetic disorders that cause immune dysfunction and predisposition to infection. Selective susceptibility to weakly virulent mycobacteria, such as *M. bovis* Bacillus Calmette-Guerin vaccine or environmental mycobacteria species, is a genetically heterogeneous group of rare PIDs so far associated with mutations in nine genes (*IFNGR1*, *IFNGR2*, *IL12B*, *IL12RB1*, *STAT1*, *ISG15*, *IRF8*, *IKBKG* and *CYBB*)[1]. These mutations impair the production of or the response to a cytokine Interferon-γ (IFN-γ), either directly or indirectly, indicating that the IFN-γ pathway is critical for the confinement of mycobacterial infection[2]. Nevertheless, genetic aetiology in approximately half of patients with Mendelian susceptibility to mycobacterial diseases remains unknown[3].

IFN-γ is a type II interferon that binds to the IFN-γ receptor, a heterodimer encoded by genes *IFNGR1* and *IFNGR2*. Stimulation of the IFN-γ receptor results in the downstream activation of two Janus kinases: JAK1 and JAK2. Upon activation, JAKs trans-phosphorylate each other at tyrosines within the kinase domain and phosphorylate the cytoplasmic tail of the receptor[4]. This allows recruitment of the Signal Transducer and Activator of Transcription 1 (STAT1) protein, which in turn is phosphorylated, forms homodimers, relocates to the nucleus, binds the Gamma Activated Sequences in the genome and drives the expression of genes implicated in cellular immunity, including antigen processing and presentation and activation of microbicidal effector functions. Intracellular signalling of type I interferons, for example, IFN-α and IFN-β, is mediated by the Interferon-α receptor encoded by *IFNAR1* and *IFNAR2*. The receptor interacts with Janus kinases JAK1 and TYK2, leading to phosphorylation of STAT1 and STAT2 proteins, which then form a heterodimer that translocates to the nucleus, forms a complex with Interferon Regulatory Factor 9 and induces the expression of the interferon-stimulated genes[5]. Multiple other cytokine receptors also signal through combinations of four JAKs and seven STAT proteins, for example, JAK1 is also used in signalling by IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, IL-27, IL-6 family cytokines and IL-10 family cytokines[4]. To date, germline mutations in two out of the four known Janus kinases, JAK3 and TYK2, have been found in PID patients[6–9]. Somatic mutations in JAK2 have also been shown to cause clonal myeloproliferative disorders, for example, polycythemia vera and idiopathic erythrocytosis[10,11], whereas somatic JAK1 mutations have been associated with gynaecologic cancers[12].

Here, we report the identification of germline *JAK1* mutations that result in a functional JAK1 deficiency associated with susceptibility to atypical mycobacterial infection and early-onset bladder carcinoma. Furthermore, detailed analyses of the mutant protein reveal phosphorylation-independent mechanism of JAK1 in signal transduction.

Results

Immunodeficiency with susceptibility to mycobacteria. We studied a 22-year-old male of Pakistani descent, the last of four children born to a consanguineous marriage of first cousins (Fig. 1a). The patient presented to paediatric immunology at the age of 3 years with a history of global developmental delay and recurrent ear and chest infections that started during the first year of life and required multiple hospital admissions. The patient had received childhood vaccines—including Bacillus Calmette-Guerin vaccine at birth—and had normal-course chicken pox at age 3 with one subsequent episode of shingles. During examination, a skeletal survey demonstrated lytic and sclerotic lesions affecting long bones, vertebrae and facial bones. The patient also developed cervical lymphadenopathy. Bone biopsy was unremarkable, lymph node biopsy reactive and no pathogen was cultured from either tissue. Considering that these features were associated with failure to thrive, raised erythrocyte sedimentation rate (ESR) (70–90 mm per hr), elevated polyclonal IgG (25–30 $g\,l^{-1}$), platelets (600–700 $\times 10^9\,l^{-1}$) and white cell count (20–25 $\times 10^9\,l^{-1}$), he was further investigated for infection. Mycobacterial skin tests for *Mycobacterium avium and Mycobacterium intracellulare* were negative, but *Mycobacterium tuberculosis (Mtb)*, *Mycobacterium malmoense* and *Mycobacterium scrofulaceum* skin tests were all strongly positive. As his relatively indolent clinical course was not typical for tuberculosis and *Mtb* had not been cultured from bone or lymph node, a clinical diagnosis of systemic atypical mycobacterial infection was made. He received anti-mycobacterial treatment (Isoniazid, Ethambutol and Ciprofloxacin, as other agents were not tolerated) and his condition improved over 12 months with catch-up growth (from 3rd to 25th centile), weight gain (25th to 50th centile) and improvement in ESR, IgG, platelets and white cell count. His bone X-rays also showed improvement with residual vertebral collapse, supporting a diagnosis of resolving multifocal osteomyelitis caused by mycobacterial infection. The immunology investigations demonstrated normal numbers of T and B cells, reduced populations of naive CD4+ and CD8+ T cells (Table 1) with normal proliferation after phytohemagglutinin (PHA) stimulation, and mildly reduced responses to Candida and purified protein derivative antigens. Total IgG and IgA levels were increased, whereas IgM level was normal, as were specific antibody responses after tetanus, Hib and pneumococcal vaccinations. The karyotype, metabolic screen and chromosomal radio-sensitivity assays were normal.

The patient remained relatively well until the age of 16, with mild developmental delay and short stature. His IgG levels remained high and over time IgM levels fell below the normal range (Table 1) with persistent mild T lymphopenia and impaired responses to PHA stimulation. Normal CDR3 spectratyping results in CD4+ and CD8+ T cells were found, with all TCR Vβ families represented with a Gaussian distribution. T-cell receptor excision circles levels in CD4+ and CD8+ T cells were normal.

At the age of 16 years the patient presented with unexplained cardiomyopathy and a raised ESR (20–40 $mm\,h^{-1}$) and was found to have a mediastinal mass on computed tomography imaging. Biopsies showed pleural and mediastinal fibrosis with patches of macrophage infiltration in lung tissue. No granulomas were seen and Quantiferon TB Gold test was negative. *Mycobacterium gordonae* was isolated from a single sputum sample, but its relevance remained unclear. In view of his previous history he received empiric treatment for atypical mycobacteria (Rifampicin and Ethambutol, Clarithromycin and Ciprofloxacin) with improvement of ESR, IgG level and the mass but permanent presumed fibrotic occlusion of the right pulmonary vein. He remained on long-term prophylaxis with Clarithromycin and Ciprofloxacin and had no recurrence or further mycobacterial infections. He had a number of skin infections, including planar warts restricted to the forehead, presumed fungal infections of his nails and severe Norwegian scabies.

At the age of 21 years the patient developed significant anaemia. He had a history of intermittent red blood per rectum and no recent history of haematuria. Colonoscopy revealed a large sessile polyp in his rectum, which was histologically benign without dysplasia. Thickening of the bladder wall was noted on magnetic resonance imaging and an extensive fungating tumour was observed on cystoscopy. Biopsies of the tumour and a supraclavicular lymph node confirmed

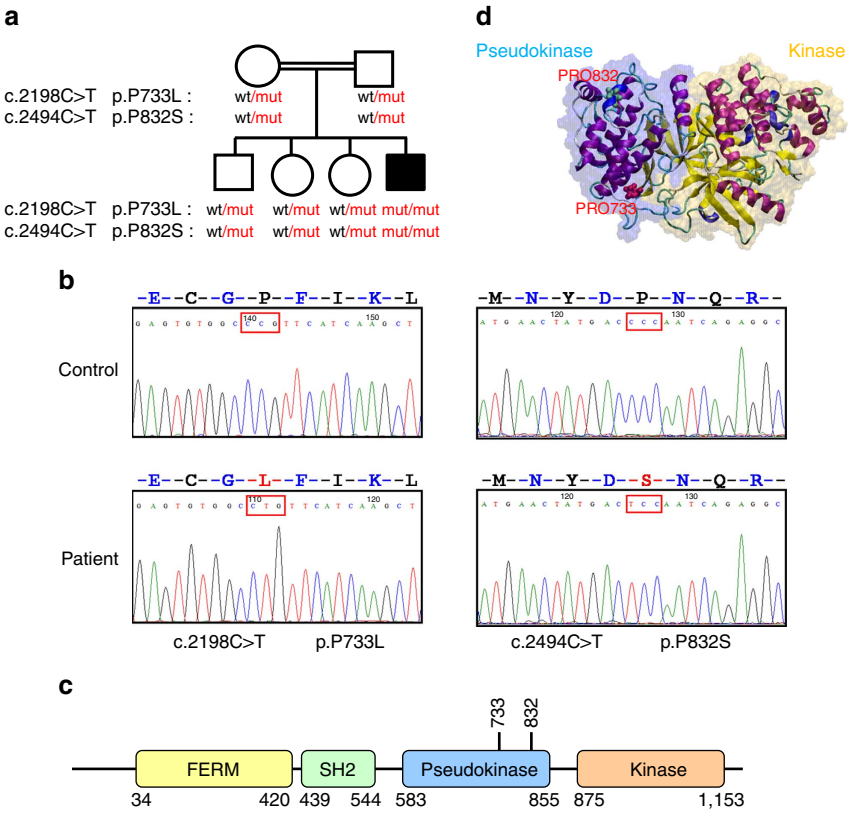

Figure 1 | Two JAK1 mutations found in the patient. (**a**) Patient's family tree. (**b**) Sequence chromatograms showing two mutations in the JAK1 gene. (**c**) Domain structure of the JAK1 protein. (**d**) JAK1 pseudokinase (JH2) and kinase (JH1) domains modelled using the published structure of TYK2 pseudokinase and kinase domains (PDB 4OLI). Pro733 and Pro832 are shown in red and green, respectively.

high-grade metastatic transitional cell carcinoma. The patient received treatment with chemotherapy including cisplatin and gemcitabine, but died aged 23 years.

Exome sequencing identifies homozygous *JAK1* mutations. As the patient was born to a consanguineous family, we hypothesized that his disease was caused by a recessive Mendelian mutation and investigated this possibility by whole-exome sequencing. The exome data contained 18,029 single nucleotide variants and small insertions/deletions, including 230 very rare ones that were not observed in the 6,500 NHLBI Exomes[13], 1,000 Genomes database (April 2012 data release)[14] and 2,500 exomes analysed internally using the same bioinformatics pipeline. Five of these rare variants were homozygous (Supplementary Table 1). Of these five, three were predicted to be benign, whereas two missense mutations were predicted to be probably damaging; both were located in the *JAK1* gene, leading to amino-acid changes from proline to leucine (p.P733L) and from proline to serine (p.P832S) (Fig. 1b). We found no mutations in genes previously associated with Mendelian susceptibility to mycobacterial disease, or other genes involved in the JAK-STAT signalling pathways. We confirmed both *JAK1* mutations by Sanger sequencing (Fig. 1b) and found that unaffected parents and all three siblings were heterozygous carriers of both mutations (Fig. 1a). We then designed genotyping assays for both *JAK1* mutations, screened 1,050 healthy subjects representing 51 populations from around the world[15] and found no healthy carriers. The ExAC database that contains exome data from > 60,000 subjects had no instance of the p.P733L mutation, whereas p.P832S was detected in four heterozygous individuals (frequency = 0.000033).

Similarly to other Janus kinases, JAK1 has FERM and SH2 domains that are responsible for interaction with the cytokine receptor, the pseudokinase (JH2) domain that regulates kinase activity, and the kinase (JH1) domain[16,17]. The proline residues at JAK1 positions 733 and 832 are located in the pseudokinase domain (Fig. 1c). They are conserved within the human Janus kinase family and in JAK1 across species (Supplementary Fig. 1). We visualized both mutations by modelling JAK1 pseudokinase and kinase domains on the published TYK2 structure[18]. Although P832S was located far from the kinase domain, P733L mapped in the β7–β8 loop close to the inter-domain interface and may affect interaction between the domains (Fig. 1d). Taken together, these results suggest that the identified JAK1 genetic variants, P733L in particular, could be recessive pathogenic mutations rather than rare neutral polymorphisms.

Multiple affected JAK1-mediated pathways in immune cells. To test the hypothesis that JAK1 P733L and P832S mutations are pathogenic, we studied STAT phosphorylation in the patient's lymphocytes (Fig. 2a). STAT1 phosphorylation was significantly reduced after IFN-α, IFN-γ and IL-27 stimulations. STAT3 phosphorylation was reduced after IL-10—but not IL-6—stimulation. Phosphorylation of STAT4 was reduced after IFN-α stimulation, as were phosphorylation of STAT5 after IL-2 stimulation and of STAT6 after IL-4 stimulation (Fig. 2a). Therefore, multiple signalling pathways mediated by JAK1 are affected in the patient's immune cells, suggesting a functional JAK1 deficiency.

Table 1 | Immunological investigations.

Cell type/Ig class	Patient (3 years 8 months)	Age-matched control range	Patient (10 years)	Age-matched control range	Patient (19 years)	Age-matched control range
White cell count	*$26.8 \times 10^9\,l^{-1}$	$5.0\text{–}15.0 \times 10^9\,l^{-1}$	$4.95 \times 10^9\,l^{-1}$	$4.5\text{–}13.5 \times 10^9\,l^{-1}$	$7.8 \times 10^9\,l^{-1}$	$4\text{–}11.9 \times 10^9\,l^{-1}$
Neutrophil count	*$17.78 \times 10^9\,l^{-1}$	$1.0\text{–}8.5 \times 10^9\,l^{-1}$	$2.47 \times 10^9\,l^{-1}$	$1.8\text{–}8.0 \times 10^9\,l^{-1}$	$4.9 \times 10^9\,l^{-1}$	$2\text{–}7.5 \times 10^9\,l^{-1}$
Lymphocyte count	$6.08 \times 10^9\,l^{-1}$	$3.0\text{–}13.5 \times 10^9\,l^{-1}$	$1.62 \times 10^9\,l^{-1}$	$1.1\text{–}5.9 \times 10^9\,l^{-1}$	$1.55 \times 10^9\,l^{-1}$	$1\text{–}2.8 \times 10^9\,l^{-1}$
CD3+ T cells	49%, $3.0 \times 10^9\,l^{-1}$	39–73%, $1.8\text{–}8.0 \times 10^9\,l^{-1}$	*45%, $0.73 \times 10^9\,l^{-1}$	55–78%, $0.7\text{–}4.2 \times 10^9\,l^{-1}$	*29%, *$0.45 \times 10^9\,l^{-1}$	55–83%, $0.7\text{–}2.1 \times 10^9\,l^{-1}$
CD19+ B cells	*45%, $2.7 \times 10^9\,l^{-1}$	17–41%, $0.6\text{–}3.1 \times 10^9\,l^{-1}$	*33%, $0.53 \times 10^9\,l^{-1}$	10–31%, $0.2\text{–}1.6 \times 10^9\,l^{-1}$	*40%, $0.62 \times 10^9\,l^{-1}$	6–19%, $0.1\text{–}0.5 \times 10^9\,l^{-1}$
CD16+CD56+ NK cells	3%, $0.2 \times 10^9\,l^{-1}$	3–16%, $0.1\text{–}1.4 \times 10^9\,l^{-1}$	20%, $0.32 \times 10^9\,l^{-1}$	4–26%, $0.09\text{–}0.9 \times 10^9\,l^{-1}$	28%, $0.43 \times 10^9\,l^{-1}$	7–13%, $0.09\text{–}0.6 \times 10^9\,l^{-1}$
CD3+CD4+ T cells	28%, $1.7 \times 10^9\,l^{-1}$	25–50%, $0.9\text{–}5.5 \times 10^9\,l^{-1}$	23%, $0.37 \times 10^9\,l^{-1}$	27–53%, $0.3\text{–}2.0 \times 10^9\,l^{-1}$	*16%, *$0.25 \times 10^9\,l^{-1}$	28–57%, $0.3\text{–}1.4 \times 10^9\,l^{-1}$
CD3+CD8+ T cells	23%, $1.4 \times 10^9\,l^{-1}$	11–32%, $0.4\text{–}2.3 \times 10^9\,l^{-1}$	16%, $0.26 \times 10^9\,l^{-1}$	19–34%, $0.3\text{–}1.8 \times 10^9\,l^{-1}$	12%, *$0.19 \times 10^9\,l^{-1}$	10–39%, $0.2\text{–}0.9 \times 10^9\,l^{-1}$
CD4+CD45RA+ T cells	*12%	62–90 %			43%†	31–65%
CD8+CD45RA+ T cells	*9%	46–85 %			*24%†	42–73%
γδT-cell	2%	<10%				
IgG	*$38.8\,g\,l^{-1}$	$3.1\text{–}13.8\,g\,l^{-1}$	*17.8	5.4–16.1	$11.9\,g\,l^{-1}$	6.0–16.0
IgA	*$1.8\,g\,l^{-1}$	$0.3\text{–}1.2\,g\,l^{-1}$	1.00	0.7–2.5	$0.9\,g\,l^{-1}$	0.8–2.8
IgM	$1.5\,g\,l^{-1}$	$0.5\text{–}2.2\,g\,l^{-1}$	0.61	0.5–1.8	*$0.45\,g\,l^{-1}$	0.5–1.9
IgG1			*12.9	3.6–7.3		
IgG2			*0.82	1.4–4.5		
IgG3			0.77	0.3–1.1		
PHA stimulation‡	172	≥70	*12.6	≥70	*3.32	≥14.4
Candida stimulation‡	*17.6	≥26.8				
PPD stimulation‡	*26.2	≥41.5				
CD3 stimulation‡					*3.2	≥7.6

*Denotes an abnormal result.
†Results are shown for the CD45RA+CD27+ cells.
‡T-cell stimulation index calculated as maximum stimulation value/background value.

We then stimulated whole blood from the patient and measured production of cytokines IFN-γ, IL-12, IL-10, TNF-α and IL-6. In assays normalized for T-cell counts we found consistently low IFN-γ production in response to PHA stimulation and after co-stimulations with PHA/IL-12 or PHA/IFN-α in comparison with healthy controls (Fig. 2b). However, upregulation of IFN-γ production after co-stimulations with lipopolysaccharide/interleukin-12 (LPS/IL-12) or LPS/IFN-α was normal. Also, the patient had low IL-10 production after PHA stimulation and co-stimulation with PHA/IFN-α (Fig. 2c). Production of IL-6 and TNF-α was increased after LPS stimulation (Fig. 2d,e), whereas production of IL-12 was normal (Fig. 2f). These results indicate that functional JAK1 deficiency is characterized by a broad immune dysregulation.

Impaired phosphorylation of JAK1, partner JAKs and STATs. To understand how the P733L and P832S mutations affect JAK1, we next looked at IFN-α and IFN-γ signalling in patient's primary fibroblasts. We first tested the hypothesis that mutations impact on the stability of the JAK1 protein, affecting, in turn, its intracellular levels. We measured JAK1 in fibroblasts derived from the patient or from two healthy subjects (Supplementary Fig. 2a), and in HEK-239T cells transiently expressing the wild type $JAK1^{WT}$, the patient-derived double-mutant $JAK1^{P733L/P832S}$ or the kinase-dead mutant $JAK1^{K908E}$ (Supplementary Fig. 2b). The patient-derived variant of JAK1 was expressed at a slightly lower level than the wild-type JAK1.

The JAK pseudokinase domain regulates activity of the kinase domain. Hence, both engineered and naturally occurring mutations in the pseudokinase domain of various JAKs, including known somatic mutations in JAK1, can affect the kinase activity[19–22]. To investigate functional effects of the P733L and P832S mutations, we measured JAK1-mediated activation of STAT1 and STAT2 proteins in response to stimulation with cytokines IFN-α and IFN-γ. The level of phosphorylation of JAK1 upon exposure to IFN-α was profoundly reduced in patient-derived fibroblasts as compared with control cells (Fig. 3a). Phosphorylation of STAT1 and STAT2 was also impaired (Fig. 3a). A reduced STAT1 phosphorylation was also observed upon treatment with IFN-γ (Fig. 3b). The induction of expression of interferon-regulated genes was also lower in the patient fibroblasts compared with control fibroblasts (Fig. 4 and Supplementary Fig. 3). These data demonstrate that not only patient's peripheral blood mononuclear cells, but also primary fibroblasts show impaired JAK1 functions, leading to reduced downstream STAT signalling; such fibroblasts therefore provide a suitable model for the analysis of JAK1 functions.

The JAK1 pseudokinase domain keeps the basal activity of the kinase in check and mediates the cytokine-inducible activation of signalling[20]. The mutations found in the patient could either affect the level of JAK1 phosphorylation or its onset or decay, causing delayed or shortened cellular responses. We studied responses to IFN-α and IFN-γ in a time-course experiment and observed reduced levels of phosphorylated JAK1, as well as STAT1, with no effect on the duration or the steepness of the activation/inactivation phases (Fig. 5a,b). These results suggest that the phospho-transfer function of the mutant JAK1 was impaired, whereas the basal kinetics of phosphorylation and dephosphorylation were normal.

We then studied if the mutant JAK1 affected phosphorylation of the partner Janus kinases TYK2 and JAK2 after stimulation with IFN-α and IFN-γ, respectively. We found that in patient's fibroblasts the amount of phosphorylated TYK2 was strongly reduced, whereas the amount of phosphorylated JAK2 was only slightly diminished (Fig. 6).

P733L has stronger effect on signalling than P832S. To study effects of each of the two patient's mutations separately, we cloned the wild-type JAK1 (JAK1^{WT}) and introduced P733L, P832S or P733L/P832S mutations by site-directed mutagenesis. We then expressed these constructs in the human fibrosarcoma U4A cells that lack endogenous JAK1 (ref. 23). The robustness of this model relies on the fact that STAT1 and STAT2 phosphorylation after stimulation with interferons is totally

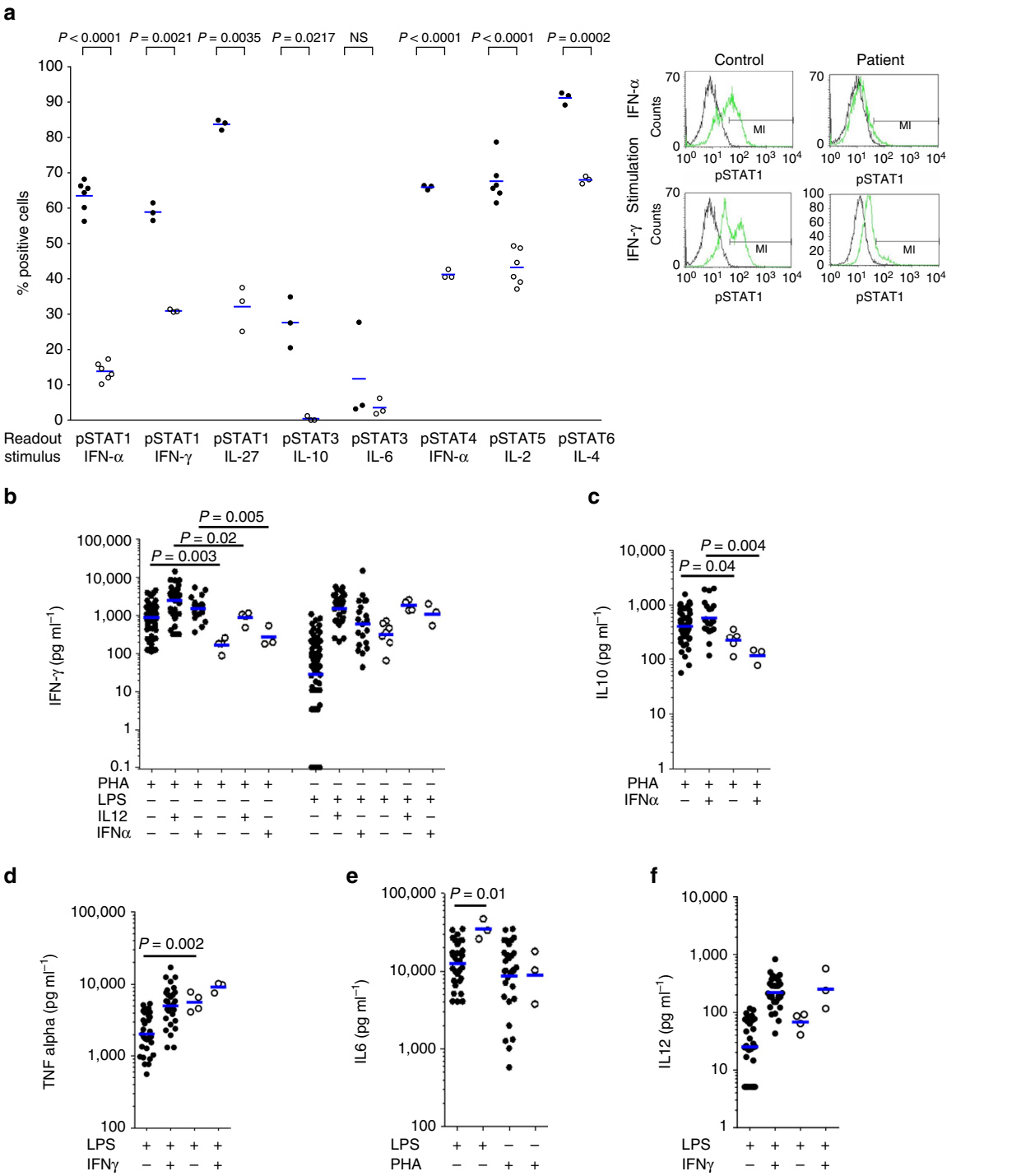

Figure 2 | Impaired STAT phosphorylation and cytokine responses in the patient's blood cells. (**a**) Left panel. Numbers of cells positive for the presence of phosphorylated STAT proteins were measured by FACS after 10 min stimulation of whole blood of the patient (age 20 years) (open circles) and compared with a healthy travel control tested under the same conditions (black dots). The assay was repeated either three times or six times (thrice on two occasions, in which case two different travel controls were studied). Blue lines show geometric means. Unpaired two-tailed Student *t*-test with Welch's correction. Right panel. FACS gating for pSTAT1 after IFN-α and IFN-γ stimulation is shown. (**b**–**f**) Cytokine responses measured after stimulation in whole blood of the patient in independent assays (open circles) and compared with healthy controls tested under the same conditions (black dots). Numbers of controls in different assays were: (**b**) LPS only $n=65$, LPS + IL-12 $n=40$, LPS + IFN-α $n=20$, PHA only $n=60$, PHA + IL-12 $n=45$, PHA + IFN-α $n=20$; (**c**) PHA only $n=50$, PHA + IFN-α $n=20$; (**d**–**f**) $n=30$. Blue lines show geometric means. Two-tailed Mann–Whitney test.

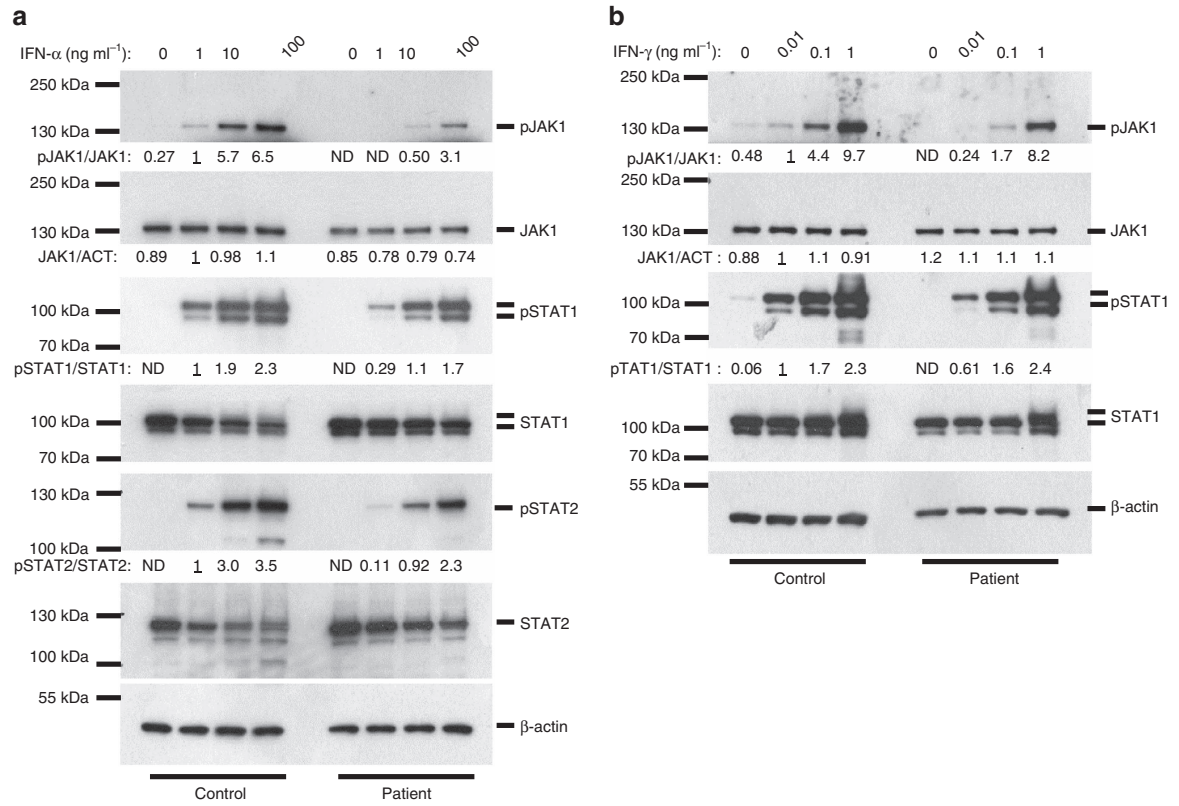

Figure 3 | Impaired JAK1-mediated signalling in the patient's fibroblasts. (**a**,**b**) Primary fibroblasts from the patient or a healthy control were treated with the indicated concentrations of IFN-α (**a**) or IFN-γ (**b**) for 15 min and protein extracts were subjected to immunoblotting. Representative of three independent experiments. Fold change of band densitometry is indicated (numbers below bands and bar graphs in Supplementary Fig. 6).

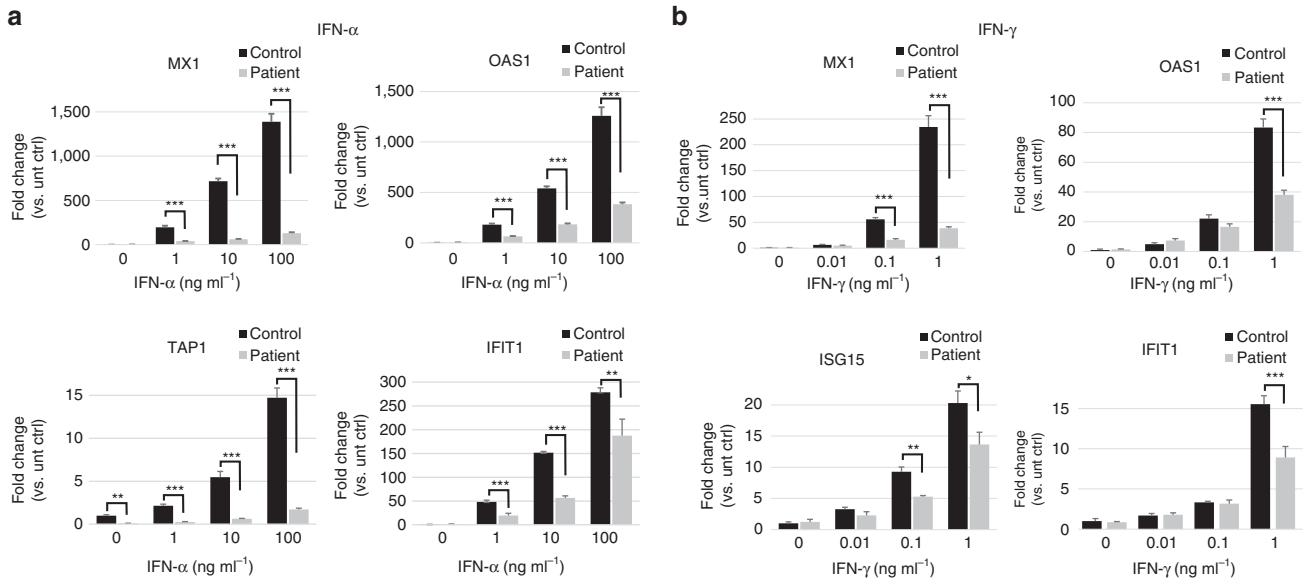

Figure 4 | Patient's fibroblasts show reduced induction of gene expression after IFN-α and IFN-γ stimulation. Cells were stimulated with IFN-α for 15 h (**a**) or IFN-γ for 8 h (**b**). qPCR was done in triplicate. mRNA fold change is shown relative to the untreated control fibroblasts. $*P<0.05$, $**P<0.005$, $***P<0.0005$. Graphs show mean values ± s.d.

dependent on JAK1 and in U4A cells the signalling pathway is rescued only upon re-expression of JAK1 (Fig. 7a,b)[23,24]. We found that phosphorylation of JAK1^{P832S} was similar to that of JAK1^{WT}, but phosphorylation of JAK1$^{P733L/P832S}$ was completely abolished and that of JAK1^{P733L} was either abolished (Fig. 7a) or very strongly reduced (Fig. 7b), suggesting that the amino-acid change P733L in the pseudokinase domain impairs JAK1 function leading to the reduced phosphorylation of tyrosines Y1034 and Y1035 in the activation loop of the kinase domain (Fig. 7a,b). Nevertheless, all four JAK1 variants were able to mediate STAT1 and STAT2 phosphorylation (Fig. 7a,b).

We then transduced primary patient fibroblasts with lentiviral vectors expressing the wild-type and mutant JAK1 proteins (Fig. 7c). Forced expression of JAK1^{WT} significantly increased

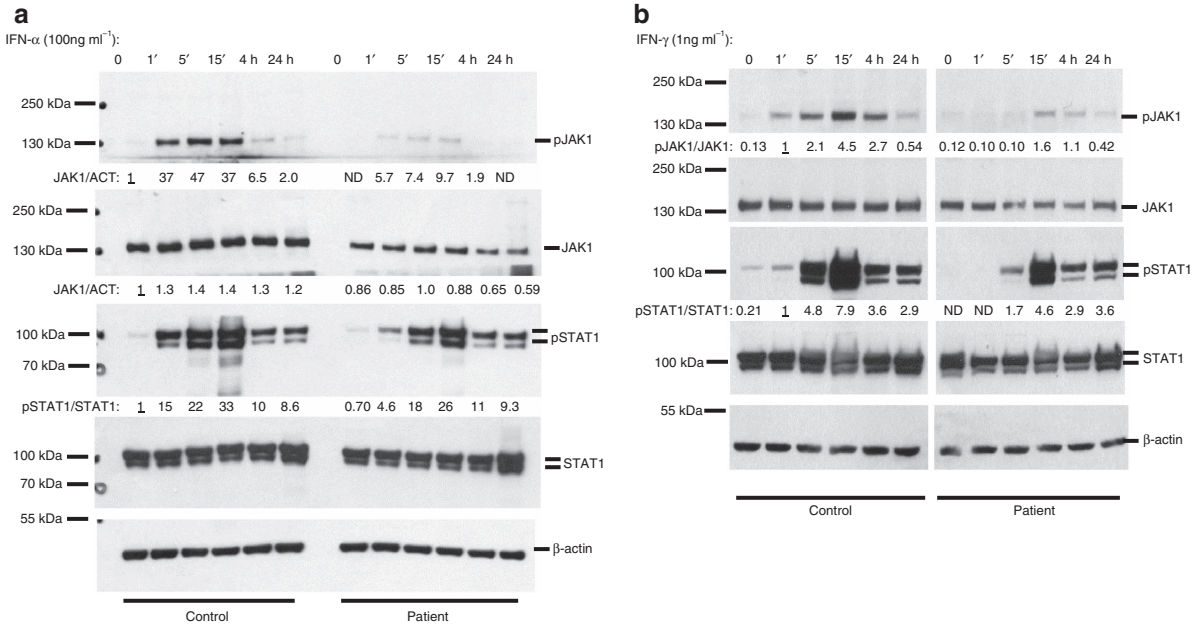

Figure 5 | Reduced JAK1 and STAT1 phosphorylation in patient's fibroblasts at different time points after IFN-α and IFN-γ stimulation. (**a**,**b**) Primary fibroblasts from the patient or a healthy control were treated with the IFN-α (**a**) or IFN-γ (**b**) for indicated times and protein extracts were subjected to immunoblotting. Representative of two independent experiments. Fold change of band densitometry is indicated (number below bands and bar graphs in Supplementary Fig. 7).

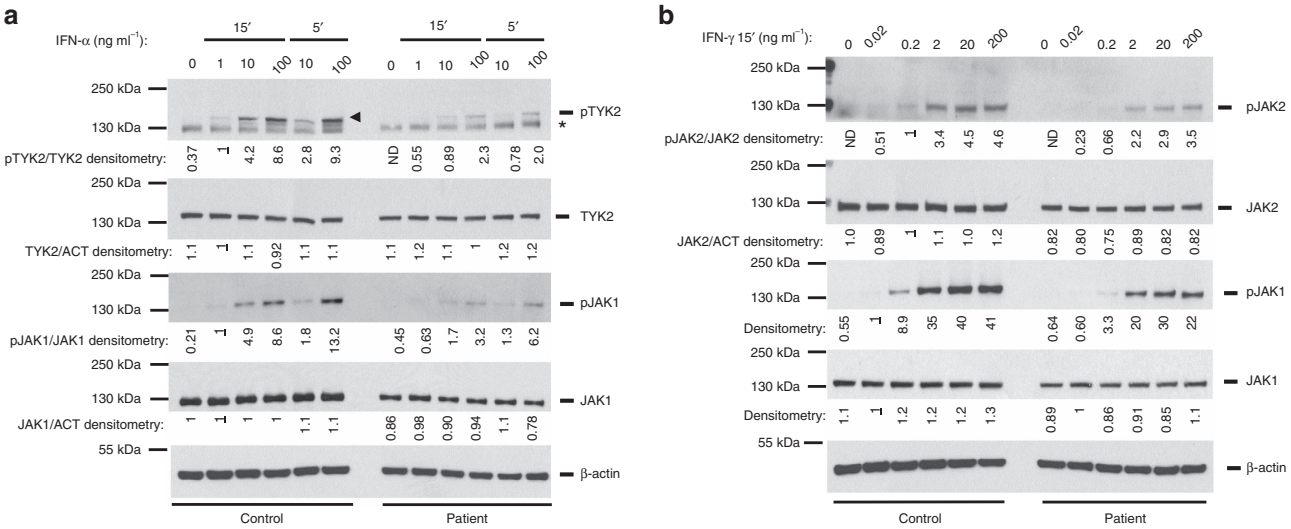

Figure 6 | Reduced amounts of phosphorylated TYK2 and JAK2 in the patient's fibroblasts. (**a**,**b**) Primary fibroblasts from the patient or a healthy control were treated with the indicated concentrations of IFN-α (**a**) or IFN-γ (**b**) and protein extracts were subjected to immunoblotting. *shows non-specific protein species detected by the antibody. Arrowhead shows specific bands. Representative of three independent experiments (IFN-α 15′ and IFN-γ) or one experiment (IFN-α 5′). Fold change of band densitometry is indicated (numbers below bands and bar graphs in Supplementary Fig. 8).

STAT1 phosphorylation after stimulation with IFN-α and IFN-γ. Forced expression of JAK1^{P832S} rescued STAT1 phosphorylation to a similar extent as JAK1^{WT}, whereas expression of the JAK1^{P733L} or JAK1$^{P733L/P832S}$ proteins led to significantly reduced STAT1 phosphorylation. Taken together, these results indicate that the double-mutant JAK1$^{P733L/P832S}$ protein is functionally deficient and that its defect is mostly caused by the P733L mutation, whereas the contribution of P832S is less pronounced.

JAK1 can mediate signalling independently of its phosphorylation. It is known that overexpression of JAKs can lead to self-phosphorylation and activation[25]. This explains the observation that forced expression of JAK1^{WT} or JAK1^{P832S} in U4A cells caused spontaneous self-phosphorylation of JAK1, as well as phosphorylation of STAT1 and STAT2, even in the absence of interferon stimulation (Fig. 7a,b). Unexpectedly we observed that following interferon stimulation, JAK1$^{P733L/P832S}$ was able to induce STAT1 and STAT2 phosphorylation even in the absence of JAK1 phosphorylation (Fig. 7a,b). Similarly, JAK1^{WT} and JAK1^{P832S} could induce STAT1 and STAT2 phosphorylation in a dose-dependent manner in response to interferon stimulation, despite the level of JAK1 phosphorylation remaining relatively constant (Fig. 7a,b). These observations suggest that JAK1 has a mode of function in interferon signalling that is independent of

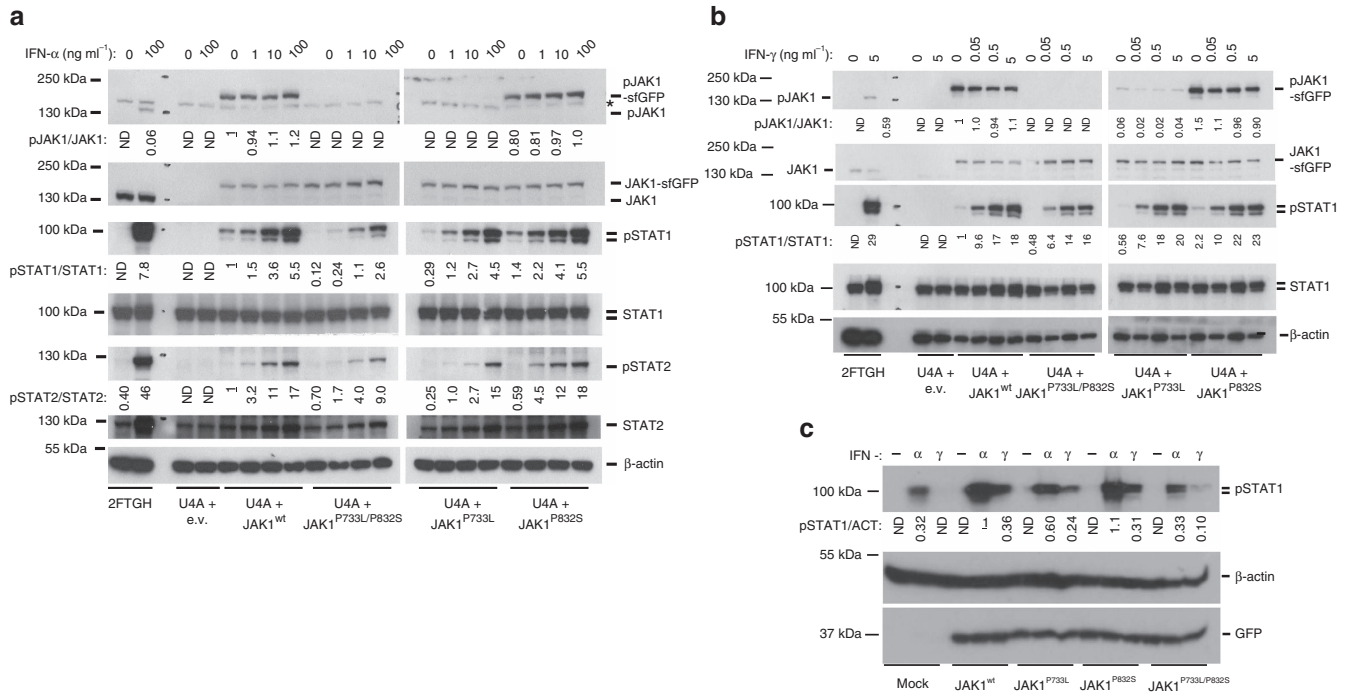

Figure 7 | P733L mutation impairs JAK1 function. (**a**,**b**) JAK1-deficient fibrosarcoma cells, U4A, were transfected for 24 h with vectors expressing sfGFP-fused JAK1^{WT}, JAK1^{P733L}, JAK1^{P832S} or JAK1$^{P733L/P832S}$. Then, cells were exposed to the indicated concentrations of IFN-α (**a**) or IFN-γ (**b**) for 15 min. Cells transfected with empty vector (e.v.) and parental, JAK1-competent, fibrosarcoma cells 2fTGH, served as negative and positive controls, respectively. *shows non-specific protein species detected by the antibody. Representative of four independent experiments. Fold change of band densitometry is indicated (numbers below bands and bar graphs in Supplementary Fig. 9).(**c**) Patient fibroblasts were transduced with vectors expressing JAK1^{WT}, JAK1^{P733L}, JAK1^{P832S} or JAK1$^{P733L/P832S}$ and eGFP. Cells were stimulated with either IFN-α (100 ng ml^{-1}) or IFN-γ (1 ng ml^{-1}) or medium (−). One experiment. Fold change of band densitometry is indicated (numbers below bands).

phosphorylation of tyrosines in its activation loop, for example, providing a scaffold for the juxtaposed Janus kinase. This putative JAK1 function could explain why such a profound defect in JAK1 phosphorylation observed in the patient-derived fibroblasts leads to only modest reduction in the downstream STAT phosphorylation (Figs 3 and 5).

JAK1 pseudokinase domain is essential in IFN-γ signalling. To further study this hypothesis and to identify the domain responsible for kinase-independent JAK1 function, we cloned a series of its superfolder green fluorescent protein (sfGFP)-tagged kinase-dead and deletion mutants (JAK1^{K908E}, JAK1^{KinΔ}, JAK1^{ΨKinΔ}, JAK1$^{ΨKinΔ/K908E}$, JAK1$^{KinΔ/ΨKinΔ}$; Supplementary Fig. 4) and studied them alongside the patient mutation (JAK1$^{P733L/P832S}$). To ensure that the observed effects are not artifacts of JAK1 activation due to massive overexpression following cell transfection, we used JAK1-deficient Flp-In U4C cells[26], which, similarly to U4A, lack endogenous expression of JAK1, and generated cell clones that stably expressed our mutant JAK1 constructs inserted in the FRT sites. First, we used live imaging of these stable U4C cell clones to look at the subcellular localisation of the sfGFP-tagged mutant JAK1 proteins. The wild type and mutant JAK1 proteins were all similarly associated with cell membrane (Supplementary Fig. 5). Following IFN-α and IFN-γ stimulations, the phosphorylation of JAK1$^{P733L/P832S}$ and JAK1^{K908E} was reduced, and phosphorylation of JAK1^{ΨKinΔ} and JAK1$^{ΨKinΔ/K908E}$ was abolished (Fig. 8). After IFN-α stimulation STAT1 phosphorylation was strongly reduced in cells expressing the kinase-dead mutant JAK1^{K908E} or the mutant JAK1^{KinΔ} lacking the kinase domain; however, it was only slightly reduced in these cells after IFN-γ stimulation (Fig. 8). Cells expressing the mutant JAK1^{ΨKinΔ} protein lacking the pseudokinase domain showed strong reduction in phosphorylated STAT1 both after IFN-α and IFN-γ stimulations (Fig. 8). Complete abrogation of STAT1 phosphorylation was observed in cells expressing either JAK1$^{KinΔ/ΨKinΔ}$, which lacked both pseudokinase and kinase domains, or JAK1$^{ΨKinΔ/K908E}$, a kinase-dead mutant lacking also the pseudokinase domain. Taken together, these data demonstrate that JAK1 has a mode of function, which is independent of its kinase activity and its phosphorylation in the kinase domain, but requires the presence of its pseudokinase domain. This JAK1 function was particularly clear after IFN-γ stimulation: in the absence of its kinase domain, JAK1 with the functional pseudokinase domain can still transmit signalling after IFN-γ stimulation leading to STAT1 phosphorylation.

Discussion

Here, we describe JAK1 signalling disruptions in a patient exhibiting PID. In immune cells JAK1 mediates intracellular signalling from multiple cytokine receptors[27]. Therefore, it seems likely that impaired phosphorylation of several STAT proteins contributed to the immunodeficiency manifested by the patient. Impaired responses to IL-2 may have led to progressive T lymphopenia, whereas increased IL-6 production could have been responsible for the persistent increased serum IgG levels. JAK1-deficient mice are runted at birth and have severely reduced numbers of thymocytes, pre-B cells and mature T and B lymphocytes[28]. Although JAK1-deficient mice died perinatally, the disease in our patient has been less severe, probably because his JAK1 deficiency is partial and cells have retained ability to mediate STAT signalling. Nevertheless, the

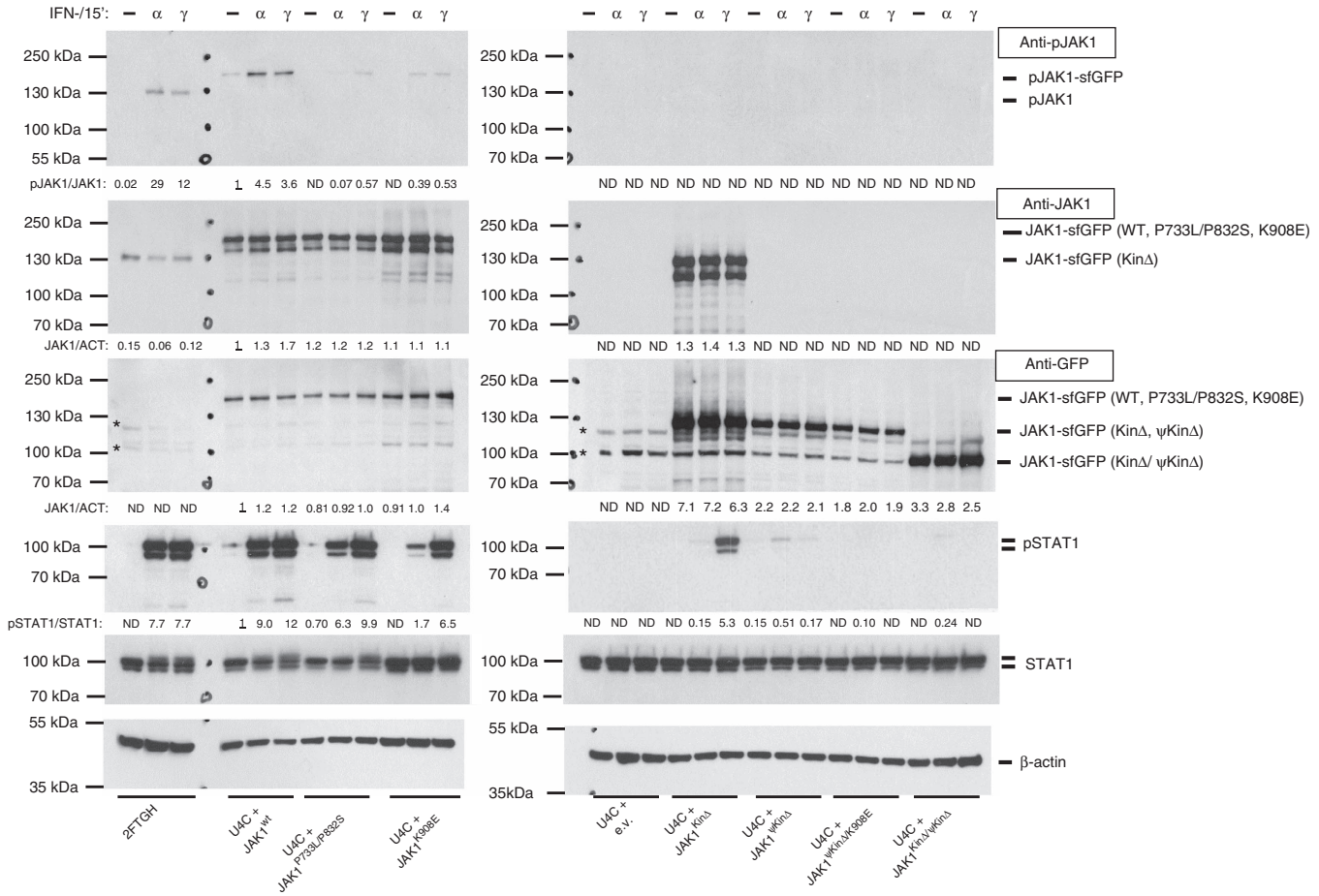

Figure 8 | JAK1 pseudokinase domain is required for mediating IFN-γ signalling. Stable clones of the JAK1-deficient Flp-In U4C fibrosarcoma cells, expressing $JAK1^{WT}$, $JAK1^{P733L/P832S}$, $JAK1^{K908E}$, $JAK1^{KinΔ}$, $JAK1^{ΨKinΔ}$, $JAK1^{ΨKinΔ/K908E}$ or $JAK1^{KinΔ/ΨKinΔ}$ were exposed to 100 ng ml^{-1} IFN-α or 5 ng ml^{-1} of IFN-γ or medium (−) for 15 min. Cells expressing empty vector (e.v.) and parental, JAK1-competent, fibrosarcoma cells, 2fTGH, served as negative and positive controls, respectively. *Shows non-specific protein species detected by the antibody. Note that anti-phospho-JAK1 antibody cannot be used to analyse phosphorylation of $JAK1^{KinΔ}$ or $JAK1^{KinΔ/ΨKinΔ}$, whereas anti-JAK1 antibody cannot detect $JAK1^{ΨKinΔ}$, $JAK1^{ΨKinΔ/K908E}$ or $JAK1^{KinΔ/ΨKinΔ}$, which lack relevant target epitopes. Representative of at least three independent experiments. Fold change of band densitometry is indicated (numbers below bands and bar graphs in Supplementary Fig. 10).

patient exhibited clinically significant susceptibility to atypical mycobacteria. This could be explained by a combination of T-cell lymphopenia, reduced IFN-γ production by the existing T cells, and impaired JAK1-STAT1 signalling downstream of the IFN-γ receptor. We found that hypomorphic JAK1 mutations have pleiotropic effects and affect multiple signalling pathways. Nevertheless, the disease manifested clinically with recurrent atypical mycobacterial infection, which suggests a dominant effect on the IFN-γ pathway.

Non-hematopoietic malignancy at a young age was a notable feature in our patient. Recently, somatic loss-of-function mutations in JAK1 have been associated with gynaecologic cancers[12]. Tumours also have been reported in patients with other defects in the IL-12/IFN-γ pathway, including Kaposi sarcoma[29], B cell lymphoma[30], disseminated cutaneous squamous cell carcinoma[31], oesophageal squamous cell carcinoma[32] and pineal germinoma[33]. These observations and the findings reported here demonstrate that impaired IL-12/IFN-γ signalling predisposes not only to mycobacterial infection, but also to malignancy, probably owing to impaired immune surveillance.

JAK1 inhibitors, for example, small molecule tofacitinib that inhibits JAK1 and JAK3, are used for treatment of rheumatoid arthritis and are currently tested in other immunological disorders, such as psoriasis and inflammatory bowel disease. Serious infections, including tuberculosis, and cancers have been reported in tofacitinib clinical trials[34–37]. These observations are in line with the clinical presentation of our patient, highlighting the phenotype associated with JAK1 deficiency, either pharmacological or genetic.

Although the JAK-STAT pathway has been studied extensively, the mechanism of JAK activation upon cytokine stimulation is not entirely clear. JAKs are activated through cytokine-induced trans-phosphorylation. It was shown that at the IL-2 receptor both JAK1 and JAK3 can trans-phosphorylate each other without being phosphorylated themselves[38]. Our data show that at the IFN-γ receptor JAK1 can transmit signalling even in the absence of its kinase domain, which precludes phosphorylation of JAK2 by JAK1. Instead, our findings suggest that JAK1 pseudokinase domain is required for interaction with JAK2, and this interaction, rather than JAK1 kinase activity, is mandatory for JAK2 activation after IFN-γ stimulation leading to STAT1 phosphorylation. In contrast, IFN-α signalling requires the presence of both kinase and pseudokinase domains of JAK1 (Fig. 8). This may be explained by hierarchical trans-activation of JAKs, where upon IFN-α stimulation JAK1 first auto-phosphorylates and then phosphorylates TYK2, whereas

upon IFN-γ stimulation it is JAK2 that auto-phosphorylates first and then phosphorylates JAK1; hence, functional JAK1 deficiency markedly impairs TYK2 phosphorylation, but has no strong effect on JAK2 phosphorylation (Fig. 6). This hierarchy is consistent with previous observations that JAK2 phosphorylation does not require active JAK1, whereas JAK1 activation requires active JAK2 (ref. 39).

Although no full-length crystal structure of JAK1 or any other Janus kinase is currently available, 2D electron microscopy averages and 3D reconstructions show that JAK1 domains have conformational flexibility and that pseudokinase and kinase domains are closely associated with each other[40]. In Janus kinases the pseudokinase domain stabilises the inactive conformation of the kinase domain[41]. In structural models of JAKs, many tumour-associated activating mutations map to the interface between the pseudokinase and kinase domains, presumably disrupting interaction between them, which facilitates activation of the kinase domain[18,41]. For example, an activating mutation F734L in JAK1, found in a sample from a T-cell acute lymphoblastic leukaemia patient[42,43], maps in the β7–β8 loop of the pseudokinase domain that interacts with the β2–β3 loop of the kinase domain[18,41]. The P733L mutation that we found in our patient maps in the same pseudokinase β7–β8 loop next to F734L, however, as we have shown here, P733L reduces JAK1-mediated signalling, suggesting that it may enhance auto-inhibitory interaction between the pseudokinase and the kinase domains.

In summary, hypomorphic recessive germline JAK1 mutations that affect multiple signalling pathways were found in a PID case that manifested with atypical mycobacterial infections and increased susceptibility to cancer. This phenotype associated with a long-term functional JAK1 deficiency predicts effects of prolonged administration of JAK1 inhibitors. We also describe a mechanism of JAK1 function in interferon signalling, which is independent of phosphorylation of tyrosines in its activation loop and its kinase function. These findings illustrate that discovery of novel naturally occurring mutations can reveal the molecular basis for human disorders, and also helps to understand fundamental biological mechanisms, even in well-characterized pathways such as the JAK-STAT pathway.

Methods

Ethics statement. Blood samples and skin biopsies were obtained with informed consent from all subjects in accordance with the Declaration of Helsinki and with approval from the ethics committee (NRES Committee London—Bloomsbury 06/Q0508/16).

Whole-exome sequencing. Library preparation, exome capture and sequencing have been done according to the manufacturers' instructions. For exome target enrichment Agilent SureSelect 38 Mb kit was used. Sequencing was done using Illumina HiSeq with 94 bp paired-end reads. Reads from raw FASTQ files were aligned to the hg19 reference genome using Novoalign version 2.08.03. Duplicate reads were marked using Picard tools MarkDuplicates. Calling was performed using the haplotype caller module of GATK (https://www.broadinstitute.org/gatk), creating genomic variant call format (gVCF)-formatted files for each sample. The individual gVCF files were combined into gVCF files containing 100 samples each. The final variant calling was performed using the GATK 'GenotypegVCFs' module jointly for all cases and controls. Variants quality scores were then re-calibrated according to GATK best practices separately for indels and SNPs. Resulting variants were annotated using software ANNOVAR.

Sequence alignment and protein modelling. The known JAK1 protein sequences from Ensembl (http://www.ensembl.org) were aligned using the Multiple Sequence Alignment ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Modelling of the pseudokinase (JH2) and kinase (JH1) domains of JAK1 was done by I-TASSER[44], using JAK1 sequence (AA 583–1153) and the three-dimensional structure of TYK2 (PDB 4OLI) as a template. The model with the best confidence score (C-Score) was visualized by Visual Molecular Dynamics software (http://www.ks.uiuc.edu/Research/vmd/).

Cells. Cell lines U4A and 2fTGH were kindly provided by Dr George Stark. Flp-In U4C (U4C-FRT) cells were kindly provided by Dr Claude Haan. Lack of JAK1 expression in JAK1-deficient U4A and U4C cells has been verified by western blotting. Cells were tested for mycoplasma contamination and were shown to be mycoplasma-negative. The parental 2fTGH human fibrosarcoma cell line and U4A and U4C-FRT cell lines, patient and control fibroblasts as well as 293T cells were cultured in DMEM medium (E15–810,PAA) and 10% heat-inactivated FCS fetal bovine serum (35 5500, Lot 001514, BD, Cowley, Oxford). Parental Flp-In U4C (U4C-FRT) cells or JAK1-complented stable clones were kept in the presence of 100 μg ml^{-1} Zeocin (Invivogen) or 250 μg ml^{-1} Hygromycin B Gold (Invivogen), respectively.

Determination of mRNA levels by relative real-time PCR. Total RNA was extracted using RNAeasy Plus Mini kit (Qiagen) from fibroblast left unstimulated or stimulated with 1–100 ng ml^{-1} IFN-α for 15 h or 0.01–1 ng ml^{-1} IFN-γ for 8 h. 0.3–1 μg RNAs were then reverse-transcribed with Oligo-dT using RevertAid Reverse Transcriptase (ThermoFisher). For the determination of the mRNA levels of the target genes, we performed qPCR using gene specific primers (Supplementary Table 2) and KAPA SYBR FAST qPCR (KK4602, Kapa biosystems). Fold changes were calculated using the ΔΔCT method against house-keeping genes.

Cloning, mutagenesis and transfection. For cloning purposes *hJAK1* gene was cloned into the vector pcDNA3.1 (+) (Invitrogen), used as a template for PCR and subcloned into a pHR-Ub-Em vector (4), using standard cloning procedures. Myc-tagged or sfGFP-tagged JAK1 constructs were subsequently cloned in pcDNA6A-myc-His (ThermoFisher, ref. V22120). Cloning of JAK1-sfGFP cDNAs in pcDNA5/FRT/TO (ThermoFisher, V652020) was performed via ApaI/KpnI sites (JAK1-sfGFP in pcDNA6A as donor plasmids). The mutations P733L, P832S, P733L/P832S and K908E were introduced using QuikChange Site-Directed mutagenesis strategy. JAK1 deletion mutants JAK1$^{\Psi Kin\Delta}$ (del aa. 583–855), JAK1$^{Kin\Delta}$ (del aa. 875–1153) and JAK1$^{\Psi Kin\Delta/Kin\Delta}$ (del aa. 583–1153) were generated via Golden Gate assembly, with outward primers carrying BsmbI (New England Biolabs) sites. Transient transfections of U4A cells were carried out using Lipofectamine reagent (ThermoFisher), following manufacturer's instructions. Stable clones of complemented U4C-FRT were generated by contrasfecting the proper JAK1-sfGFP in pcDNA5/FRT/TO construct along with pOG44 (ThermoFisher), followed by selection in Hygromycin-containing medium. The primers used in the cloning and mutagenesis are summarized in the Supplementary Table 2. sfGFP is a genetically modified form of GFP with reduced dimerisation properties in comparison with the wild-type GFP[45].

Lentivirus preparation and transductions. The lentivirus stocks were prepared by transient transfection of 293T cells (75 cm^2 flasks at 50% confluency) with: the envelope plasmid pMD.G (4 μg), the packing plasmid CMV8.91 (4 μg) and the expression plasmid pHR-UbEm (6 μg) along with 35 μl the transfection reagent Transit 2020 (5454, Mirus) following the manufacture instructions. 24 h post transfection (hpt) the media was replaced and medium was harvested at 48 and 72 h post transfection, pooled, cleared by low-speed centrifugation (1,200 rpm, 5 min), and filtered through 0.45-μm-pore-size filters, and titered by limited dilution scoring for eGFP-positive cells 3–5 days after infection. Virus stocks were stored up to 3 weeks at 4 °C for transduction experiments. Transductions of patient fibroblast and U4A cells were carried out by infection at a multiplicity of infection of 1–3 in the presence of 8 μg ml^{-1} of Polybrene (107689, Sigma) overnight, then the virus containing media was replaced by fresh media, samples were stimulated and harvested 2–5 days after infection.

Cells stimulations and immunoblotting. STATs' phosphorylation was tested in complemented U4A cells, U4C-FRT cells or patient fibroblasts along with appropriate control cells upon stimulation with IFN-α (11101–2, PBL) or IFN-γ (IF002, Millipore), in full growth medium at 37 °C. Then, the cells were trypsinized, washed and lysed in cold radioimmunoprecipitation assay buffer supplemented with phosphatases (P5726, Sigma) and protease inhibitors (11836153001, Roche) for 30 min at 4 °C. The lyses were then centrifuged at 10,000*g* for 10 min at 4 °C, the supernatants were quantified and 10–50 μg of total protein was separated by 10% SDS-PAGE and analysed by western blot. Membranes were cut horizontally according to molecular size markers, and stripes were incubated with different Abs. Immunoblots were developed with the enhanced chemiluminescence western blotting Reagent (Amersham). The following Abs were used: anti-JAK1 (610231, BD Biosciences; 1/1,000 dilution), anti-pJAK1 Tyr$^{1022/1023}$ (3331, Cell Signaling Technology; 1/1,000 dilution), anti-JAK2 (Clone D2E12, 3230, Cell Signaling Technology; 1/1,000), anti-pJAK2 Tyr$^{1007/1008}$ (Clone C80C, 33776, Cell Signaling Technology; 1:1,000), anti-TYK2 (Clone D4I5T, 14193, Cell Signaling Technology; 1:1,000), anti-pTYK2 Tyr$^{1054/1055}$ (Clone C80C, 9321, Cell Signaling Technology; 1:1,000), anti-STAT1 (9172, Cell Signaling Technology; 1:1,000), anti-pSTAT1 Tyr^{701} (Clone 58D6, 9167, Cell Signaling Technology; 1:1,000), anti-STAT2 (4594, Cell Signaling Technology; 1:1,000), anti-pSTAT2 Tyr^{689} (07–224, Millipore; 1:2,000), anti-β-Actin (Clone AC-15, A5451, Sigma-Aldrich; 1:10,000), anti-GFP (11814460001,

Roche or ab290, AbCam; 1:2,000) and anti-Myc (clone 9B11, 2276, Cell Signaling Technology; 1:1,000). Band densitometry was determined by Image Studio Lite (Licor). Fold changes of phospho/non-phosphorylated proteins were calculated against the indicated internal reference sample. Similarly, JAK1 protein levels in primary fibroblasts were determined after normalisation with β-actin (loading control). Uncropped scans of western blots are shown in Supplementary Fig. 11.

Microscopy. U4C cells complemented with sfGFP-tagged JAK1 mutants were seeded at 50,000 cell per well on 24-well Sensoplates (662892, Greiner). Two days later, living cells were rinsed twice in 1xPBS and imaged in Live Cell Imaging Solution (A14291DJ, ThermoFisher). Nuclei were stained with NucBlue reagent (R37605, ThermoFisher). Micrographs were acquired at a Leica SP5 confocal microscope.

FACS analysis of STAT phosphorylation. Blood was collected in EDTA. In total, 100 µl of blood was left unstimulated and 100 µl was stimulated with indicated cytokines for 10 min (IL-2, Chiron, 10^5 U ml^{-1}; IL-4, ImmunoTools, 1 µg ml^{-1}; IL-6, R&D systems, Abingdon, England, 100 ng ml^{-1}; IL-10, R&D systems, 500 ng ml^{-1}; IFNγ, R&D systems, 200 ng ml^{-1}; IFNα, PBL Interferon Source, 10^6 U ml^{-1}; IL-27, R&D systems, 500 ng ml^{-1}). Red cells were lysed and phosphorylation state fixed using Lyse/Fix (BD Biosciences). Cells were permeabilised with Perm Buffer III (BD Biosciences) before being stained with 5 µl surface antibodies: APC Anti-CD3 1:20 dilution, or PerCP Anti-CD4 1:20, and PE anti-STAT1 (p701) 1:20, or Alexa Fluor 488 Anti-STAT5 (Y694) 1:20, or Alexa Fluor 488 Anti-STAT4 (Y693) 1:20, or Alexa Fluor 488 Anti-STAT3 (49/STAT3) 1:20, or Alexa Fluor 488 Anti-STAT6 (Y641) 1:20 (all from BD Biosciences). Gating was done on the following lymphocyte populations: total lymphocytes for the analysis of STAT1 phosphorylation after IFN-α stimulation; CD3- lymphocytes for the analysis of STAT1 phosphorylation after IFN-γ stimulation and of STAT3 phosphorylation after IL-10 or IL-6 stimulations; CD3+ lymphocytes for the analysis of STAT4 phosphorylation after IFN-α stimulation, of STAT5 phosphorylation after IL-2 stimulation and of STAT6 phosphorylation after IL-4 stimulation; CD4+ lymphocytes for the analysis of STAT1 phosphorylation after IL-27 stimulation. The stained cells were detected using a FACsCalibur (BD Biosciences); 10,000 gated events were collected. Analysis was performed using CellQuest software (Becton Dickinson).

Whole-blood cytokine production assays. Whole blood was diluted 1:5 in RPMI into 96-well F plates (Corning) and activated by single stimulation with IL-12 (20 ng ml^{-1}; R&D Systems), PHA (10 µg ml^{-1}; Sigma-Aldrich), LPS (1 µg ml^{-1}) List Biochemicals, IFN-γ (2×10 IU ml^{-1}, Imukin, Boehringer Ingelheim), IFN-α (2×10^3 IU ml^{-1}, Intron A, Schering Plough, UK) or using co-stimulations as indicated. Supernatants were taken at 24 h. Cytokines were measured using standard ELISA according to the manufacturer's recommendations (IFN-γ, Pelikine, Sanquin, NL), or multiplexed (TNFα, IL-12, IL-10, IL-6, R+D Systems Fluorokinemap) on a Luminex analyser (Bio-Plex, Bio-Rad, UK). Data were statistically analysed by the two-tailed Mann–Whitney test using Prism 6 (GraphPad Software).

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

D.E. performed analyses of primary fibroblasts and cell lines and the structural protein analysis. I.A., F.H., M.G., K.N., V.D.-C. contributed to these analyses. S.O.B. and A.J.T. treated the patient and collected clinical data and samples. K.C.G. performed fluorescence-activated cell sorting analyses. R.D. and D.K. performed whole blood assays. V.P. and S.N. analysed exome data. J.C. performed sequencing. S.N., A.J.T. and S.O.B. supervised the study. S.N. wrote the first draft; all authors contributed to the writing of the manuscript.

Additional information

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