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Deciphering the immune response to respiratory pathogens - Role of programmed death-ligand 1

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Résumé

Les infections pulmonaires liées à des agents pathogènes respiratoires sont parmi les causes majeures de décès dans le monde entier. L'évolution de l'infection dépend de la capacité de l'hôte à répondre au défi pausé par les pathogènes. De plus, l'hôte a besoin de détecter le pathogène, de produire une réponse immunitaire efficace et à la fin stopper la réponse inflammatoire qui s'ensuit pour éviter des dommages tissulaires. Dans ce contexte, les pathogènes ont adopté un certain nombre de stratégies qui détournent les mécanismes de l'hôte pour freiner la réponse immunitaire, ce qui a pour conséquence de causer l'infection.

La vole de signalisation PDL-1 (programmed death ligand 1)-PD-1 (programmed death 1) est une voie clé impliquée dans la médiation de la tolérance du soi et par conséquent le maintien de l'homéostasie. Des articles élégants ont démontré que la voie de signalisation PD-L1 – PD-1 est utilisée par des cellules tumorales et des virus comme un mécanisme d'évasion immune pour supprimer la réponse des cellules T effectrices. Par conséquent, j'ai voulu étudier le rôle de la voie PD-L1 dans la modulation de la réponse immunitaire contre le *Mycobacterium tuberculosis*, un pathogène bactérien, et l'*Aspergillus fumigatus*, un pathogène fungique opportuniste. J'ai trouvé que l'α-(1,3)-glucan dérivé de l'*A. fumigatus* induit une maturation des cellules dendritiques (CDs) et la sécrétion de plusieurs cytokines immunorégulatrices qui sont partiellement dépendantes du Toll like receptor (TLR)-2. L'analyse de la polarisation des cellules T $CD4^+$ a révélé que les CDs éduquées par l'α-(1,3)-glucan induisent la génération de cellules T régulatrices (Treg) $CD4^+$ $CD25^+FoxP3^+$, ceci étant en partie lié à l'expression de PD-L1 sur les CDs. De façon importante, le blocage de PD-L1 sur les CDs augmente la sécrétion d'IFN-γ sans moduler la réponse Th17. De manière similaire, PD-L1 induit par *M. tuberculosis* freine la réponse Th1 sans moduler la réponse Th17. L'analyse des voies de signalisation en aval a indiqué que la voie sonic hedgehog (SHH) en réponse au mycobacterium médiait l'induction de PD-L1 en inhibant des microARNs spécifiques, miR-324-5p et miR-338-5p qui ciblent PD-L1. De plus, SHH induit la cyclooxygénase (COX)-2 qui catalyse la synthèse de la prostaglandine E_2 (PGE2) qui agit en synergie avec PD-L1 pour coordonner l'expansion des Treg. Mes résultats démontre donc que les pathogènes respiratoires soit de façon directe, soit en abritant des antigènes immunorégulateurs détournent la voie PD-L1 pour supprimer la réponse Th1 et orchestrer la génération de Tregs sans moduler la réponse Th17. De façon importante, mes résultats fournissent un rationnel pour exploiter des approches

immunothérapeutiques qui ciblent l'axe de co-stimulation PD-1 – PD-L1 pour restorer une réponse T effectrice contre les pathogènes respiratoires.

Summary

Pulmonary infections caused by respiratory pathogens are among the major causes of death worldwide. The outcome of infection depends on the ability of the host to respond to the challenge posed by the pathogens. Of note, the host needs to sense the pathogen, mount an efficient immune response and finally clear the ensuing inflammatory response to avoid tissue damage. In this context pathogens have adapted numerous strategies that hijack the host mechanisms to dampen the immune response and as a consequence causing infection.

The programmed death ligand 1 (PD-L-1) – programmed death 1 (PD-1) pathway is a key pathway involved in mediating self-tolerance thereby maintaining homeostasis. Elegant reports have demonstrated that the PD-L1 – PD-1 pathway is exploited by cancer cells and viruses as an immune evasion mechanism to suppress effector T cell responses. Thus, I aimed at investigating the role of PD-L1 pathway in modulating immune response to *Mycobacterium tuberculosis* a bacterial pathogen and *Aspergillus fumigatus* an opportunistic fungal pathogen. I found that *A. fumigatus*-derived α-(1,3)-glucan induces maturation of DCs and secretion of various immunoregulatory cytokines that was partially dependant on Toll like receptor (TLR)-2. Analysis of $CD4^+$ T cell polarization revealed that α-(1,3)-glucan-educated DCs induced $CD4^+$ $CD25^+FoxP3^+$ regulatory T cell (Treg) generation that was in part dependent on the PD-L1 expression on DCs. Importantly, blocking PD-L1 on DCs enhanced IFN-γ secretion without modulating Th17 response. Similarly, *M. tuberculosis* induced PD-L1 dampened Th1 response without modulating Th17 response. Analysis of downstream signalling pathways indicated that, mycobacterium-responsive sonic hedgehog (SHH) mediated PD-L1 induction by inhibiting specific microRNAs, miR-324-5p and miR-338-5p that target PD-L1. Additionally, SHH induced cyclooxygenase (COX)-2 catalysed the synthesis of prostaglandin E_2 (PGE2) that synergize with PD-L1 to coordinate the expansion of Tregs. My results thus demonstrate that respiratory pathogens either directly or by harbouring imuunoregulatory antigens highjack the PD-L1 pathway to suppress the protective Th1 response and orchestrate Treg generation without modulating Th17 response. Importantly, my results provide a rational for exploiting immunotherapeutic approaches that target PD-1 – PD-L1 co-stimulatory axis to restore effector T cell response to respiratory pathogens.

Table of contents

List of figures

List of table

Abbreviations

Ab	Antibody
AEC	*Airway epithelial cells*
Ag	Antigen
APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BCR	B-cell receptor
CCL	Chemokine (C-C Motif) Ligand
CD	Cluster of differentiation
CLR	C-type lectin
COX	Cyclo-oxygenase
CTL	Cytotoxic T lymphocyte
CXCL	Chemokine (C-X-C) ligand
CTLA-4	Cytotoxic T-lymphocyte-associated protein *4*
DC	Dendritic cell
ESAT-6	Early secreted antigenic target
FcγR	Fc gamma receptor
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-monocyte progenitor
HIV	H*uman immunodeficiency virus*
HSC	Hematopoietic stem cell
IA	Invasive aspergillosis
IFN	Interferron
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LAM	Lipoarabinomannan
LM	Lipomannan
LPS	Lipopolysaccharides
LAG-3	Lymphocyte-activation gene 3
MALT	Mucosal-associated lymphoid tissues
ManLAM	Mannosylated-lipoarbinomannan
MBL	*Mannose binding lectin*
MDR	Multidrug-resistant
MHC	Major Histocompability complex
miRNA	microRNA

MYD88	Myeloid differentiation primary response gene 88
NADPH	*N*icotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NLR	Nucleotide-binding oligomerization-domain protein like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization-domain
NOS	Nitric oxide synthase
PAMP	Pathogen-associated molecular patterns
PD-1	Programmed death-1
PD-L1	Programmed death-1 ligand 1
PD-L2	Programmed death-1 ligand 2
PE	Proline-Glutamate motif
PGE2	ProstaglandinE2
PGRS	Polymorphic GC-rich repetitive sequences
pH	potential hydrogen
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PP2A	Protein phosphatase 2A
PRR	Pathogen- recognition receptor
PTEN	Phosphatase and tensin homolog
RORC	Retinoid-related orphan receptor C
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
SHH	Sonic Hedgehog
SP	*Surfactant proteins*
STAT	Signal transducers and activator of transcription
Tbet	T box transcription factor
TCR	T-cell receptor
TGF-ß	Transforming growth factor- beta
Th	Helper T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells

Introduction

1 Immune system

1.1 Overview

The immune system, a fundamental structural unit of the body is a fascinating defense system that ensures survival of the host by maintaining homeostasis and providing protection against invading pathogens. It is composed of an intricate network of specialized cells, tissues, and organs. Perhaps the most remarkable feature of the vertebral immune system is the ability to distinguish non-infectious self as opposed to that of infectious non-self (foreign). This seemingly challenging process occurs by a systematic education and thorough selection in the bone marrow and the thymus, the dysregulation of which results in pathological autoimmune diseases. Nevertheless, the immune system is exceptional for its specificity.

The immune system is organized into layers with increasing specificity. In its simplest form, the physical and mechanical barrier that is constituted by the skin and the mucosal lining prevents invasion of pathogens. Pathogens that breach this layer are immediately encountered by innate cells that provide a robust immediate but non-specific responses. Lastly, pathogens that have resisted immune challenges by employing evasion mechanisms are dealt by the adaptive cells. Thus, the vertebral immune system is by far the most superior defense system that curtails invasion and pathological infection.

Functionally the vertebral immune system needs to perform four fundamental tasks.

(i) **Immunological recognition:** The primary task of the immune system is to detect the presence of infection. The process of pathogen detection is termed "immunological recognition".

(ii) **Immune effector functions:** Upon sensing, the components of immune system needs to contain and if possible completely eliminate the infection.

(iii) **Immune regulation:** Effector functions mediated by the innate and adaptive arms needs to be regulated to avoid immunopathology.

(iv) **Immunological memory:** The process by which immune system provides protection upon reinfection with the same pathogen. "Immunological memory" is a unique feature of the adaptive immune system which forms the basis for vaccine development (Janeways Immunobiology 7th edition)

1.2 Development of immune cells

All mature blood cells are originated from haematopoietic stem cells (HSCs) by a process termed haematopoiesis. It is initiated from the embryonic stage throughout adulthood. Haematopoiesis occurs in two steps, the primitive phase and the definitive phase (Galloway and Zon, 2003). The primitive phase occurs during the early embryonic development in the yolk sac. The erythrocyte progenitor which lack pluripotency gives rise to red blood cells and macrophages (Jagannathan-Bogdan and Zon, 2013; Palis et al., 1999; Palis and Yoder, 2001). The main goal of this phase is to produce red blood cells (RBCs) which support oxygenation to the growing embryo. The definitive phase is initiated in the aorta-gonad-mesonephros region (Boisset et al., 2010). During this phase, multipotent, self-renewing HSC progenitors are generated. During the early phase of gestation, haematopoiesis occurs in the foetal liver and spleen. However, postnatally haematopoiesis completely shifts to the bone marrow (Cumano and Godin, 2007).

HSCs that are formed in the bone marrow differentiate to either common lymphoid progenitor (CLP) cell or common myeloid progenitor (CMP) cell. The CLP gives rise to T cells, B cells and NK cells. The CMP gives rise to RBCs and white blood cells (WBCs) such as neutrophils, eosinophils, basophils, monocytes, macrophages, dendritic cells (DCs), mast cells and platelets (Wang and Wagers, 2011). After haematopoiesis the WBCs enters into circulation where they encounter antigens. Development and maturation of lymphocytes occur in the primary lymphoid organs - thymus and the bone marrow. Mature lymphocytes migrate to secondary lymphoid organs where they engage in antigen interaction. Lymph nodes, spleen and mucosal-associated lymphoid tissues (MALT) such as gut-associated lymphoid tissues (GALT) constitute the secondary lymphoid organs.

1.3 Innate immune system

Every multicellular organism, including plants have at least a rudimentary form of an innate immune system. It forms the first line of defense to invading pathogens. The metazoan innate immune system comprises of innate cells and soluble factors such as complement proteins. The innate cells encompass mononuclear cells - DCs, macrophages, monocytes, natural killer cell (NK), and mast cells; and the polymorphonuclear cells - eosinophils, neutrophils, and basophils. Danger signals elicited by invading pathogens and aberrant tissues are first sensed by the innate cells. The innate cells respond to pathogens by recognizing

structural patterns present on microbes by a unique set of germline-encoded receptors known as pathogen recognition receptors (PRRs) (Lemaitre et al., 1996; Medzhitov and Janeway, 2000). PRRs can recognize structurally diverse microbial targets such as complex polysaccharides, lipoproteins, glycolipids and nucleic acids that are collectively known as pathogen-associated molecular patterns (PAMPs) (Dempsey et al., 2003).

1.3.1 Pattern recognition receptors

Broadly PRRs can be categorized into secretory, transmembrane, or cytosolic. Secretory PRRs include collectins, ficolins, and pentraxins which play an important role in activating the complement system. They also mediate opsonization of pathogens and promote their phagocytosis by innate immune cells (Canton et al., 2013; Gough and Gordon, 2000). Transmembrane receptors comprise of toll-like receptor (TLRs) family proteins and C-type lectin receptors (CLRs). Mammalian TLRs are either located on the plasma membrane or in the endosomal/lysosomal organelles. Cell-surface PRRs are involved in the recognition of components present on surface of pathogens such as lipopolysaccharide (LPS) (by TLR4), flagellin (by TLR5). Endosomal PRRs are involved in the detection of microbial nucleic acids such as double-stranded RNA (dsRNA by TLR3), single stranded RNA (ssRNA by TLR7), and double-stranded DNA (dsDNA by TLR9) (Akira et al., 2006; Iwasaki and Medzhitov, 2010). CLRs harbour carbohydrate-recognition domains and thus are involved in the recognition of carbohydrates. Dectin-1 and dectin-2 are the major CLRs involved in the recognition of fungal cell wall components such as β-glucans and mannans respectively. CLRs play an important role in anti-fungal immunity (Brown, 2006; Robinson et al., 2009). Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding domain and leucine-rich repeat (LLR)-containing receptors (NLRs) constitute the cytoplasmic PRRs. RLRs are involved in the detection of ssRNA and dsRNA and play an important role during viral infections (Pichlmair et al., 2006; Yoneyama and Fujita, 2008). NLRs are intracellular sensors involved in the detection of stress signals, degraded microbial products, and non-infectious crystal particles (Martinon et al., 2009). NLRs activate caspases, which cleave inflammatory cytokines such as IL-1 and mediate the inflammatory response (Kanneganti et al., 2007; Miao et al., 2006). Most PRRs activate one of the three transcription factors: nuclear factor- kB (NF-kB), interferon regulatory factor (IRF), or nuclear factor of activated T cells (NFAT). Signalling mediated by these transcription factors is sufficient to induce an adaptive immune response.

1.3.2 Host defense mediated by innate immune system

During infection, tissue resident macrophages are among the first innate cells that encounter the pathogens. The primary function of macrophages is to eliminate the pathogen by phagocytosis. Recognition of microbial PAMPs by macrophages induces signalling of Rho GTPases (Erwig et al., 2006; Martin et al., 2014). Activation of RhoA remodels the actin cytoskeleton to engulf the pathogen in a membrane-bound vesicle called phagosome that

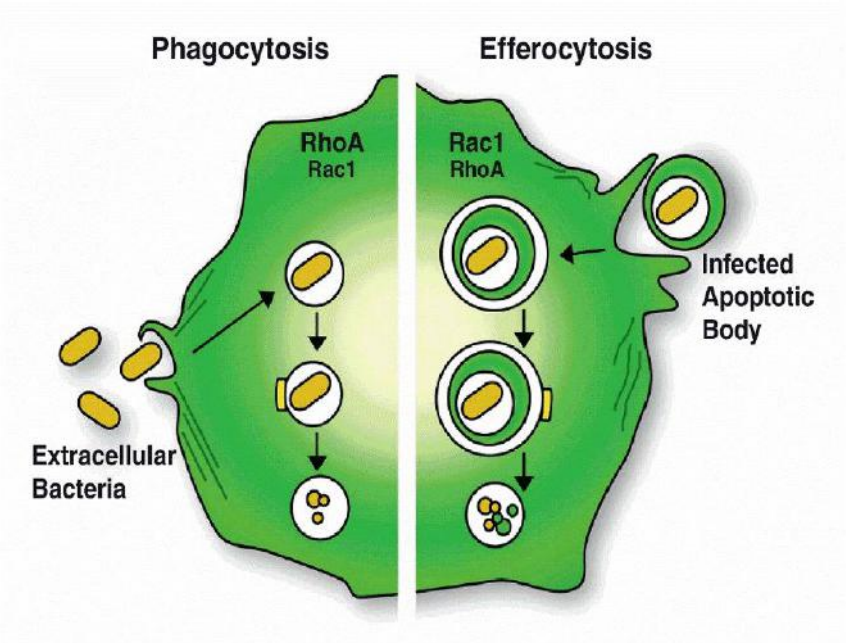

Figure 1. Phagocytosis and efferocytosis.

Increased levels of RhoA result in actin polymerization resulting in the uptake of microbes. Fusion of the actin projections leads to internalization of the bacteria in a phagosome, which undergoes stepwise maturation, leading to degradation of the pathogen. In contrast, increased levels of Rac-1 mediate efferocytosis. Rac-1 induces the formation of lamellipodia and leading to the internalization of infected and 'double-wrapped' apoptotic bodies in an efferosome. The efferosome undergoes acidification, similar to the process of phagolysosome fusion where infected or apoptotic cells are degraded. (Figure adapted from Martin CJ *et al*, 2014 Curr Opin Microbiol)

subsequently fuses with the lysosomes. Low pH and presence of hydrolytic enzymes in the lysosomes facilitates microbial degradation (Kinchen and Ravichandran, 2008). Further, macrophages maintain homeostasis by clearing apoptotic cells and infected cells by a process known as efferocytosis (Korns et al., 2011) (Fig 1). Additionally, macrophages are also involved in triggering the immune response and recruiting immune cells to the site of infection via the production of cytokines and chemokines.

Granulocytes are composed of neutrophils, basophils and eosinophils. The life span of granulocytes is short and thus are continuously replenished. IL-3, IL-5, and granulocyte macrophage-colony stimulating factor (GM-CSF) derived from immune and non-immune cells

play an important role in prolonging the survival of granulocytes (Geering et al., 2013). Neutrophils are the most abundant leukocytes and are generally considered as foot soldiers of the immune system. They are among the first responders that arrive at the site of infection/inflammation, usually within hours. Chemotactic gradients generated by vascular and tissue resident cells at the site of infection and/inflammation orchestrate neutrophil recruitment (Kim and Luster, 2015). Neutrophils mediate diverse effector function, such as phagocytosis, release of anti-microbial proteins, and generation of reactive oxygen species (ROS) that directly kill the pathogen (Kruger et al., 2015). Additionally, neutrophils undergo a novel mechanism of death called NETosis where they release extracellular DNA which trap and kill microbes (Brinkmann et al., 2004). These traps are referred to as neutrophil extracellular traps (NETs). Although the formation of NET mediates protection against pathogens, it is also implicated in autoimmune diseases and auto-inflammatory disorders (Kessenbrock et al., 2009; Sørensen and Borregaard, 2016). Eosinophils, basophils and mast cells are implicated in allergic reactions and provide protection against parasitic infection. Further, they also function as non-professional antigen presenting cells. However, human basophils lack antigen presenting features as opposed to that of murine basophils (Sharma et al., 2013; Sokol et al., 2009).

DCs were first identified in 1973 by Ralph Steinman and Zanvil Cohn from peripheral lymphoid organs of mice (Steinman and Cohn, 1973). DCs are regarded as sentinels of the immune system that bridge innate and adaptive immune system. They are formed in the bone marrow and are found in blood, lymphoid tissues, skin and mucosal surfaces. DCs are endowed with molecular sensor and antigen-processing machinery to recognize and process pathogens and/antigens through which DCs dictate the specificity, magnitude and polarity of immune response. Different subsets of DCs are known to exist which are characterized by different markers (Haniffa et al., 2013) (Fig 2).

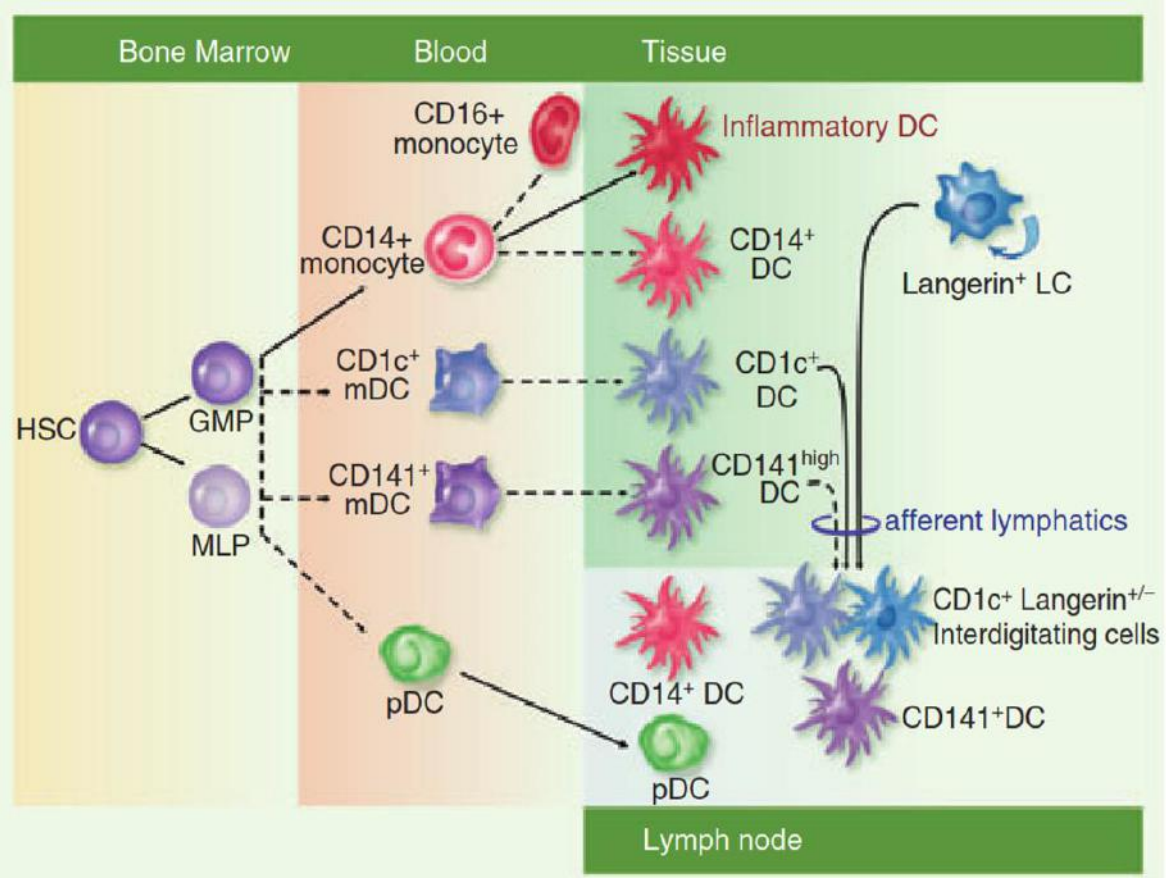

Figure 2. Distribution of major human dendritic cell (DC) subsets.

Human DCs can be generated either from granulocyte–macrophage progenitors (GMP) or multi-lymphoid progenitors (MLP) both of which ultimately arise from haematopoietic stem cells (HSC). Classical monocytes, blood myeloid DC (mDC) and plasmacytoid DC (pDC) are putative precursors of tissue and lymphoid DCs. Inflammatory DCs monocytes can give rise to inflammatory DCs. Myeloid DCs and Langerhans cells (LCs) form interdigitating cells in skin-draining lymph nodes. Broken arrows indicate relationships that require further confirmation. (Adapted from Collin et., 2013)

1.3.3 Antigen processing and presentation by dendritic cells

In homeostasis, DCs exist in an immature state. Immature DCs express low levels of major histocompatibility (MHCs) and accessory signals required for T cell activation such as CD80, CD86, and CD40. However, functionally immature DCs have a high endocytic activity to sample antigens. Captured antigens are processed for antigen presentation by MHC-I or MHC-II. MHC-I is present in all nucleated cells, in contrast MHC-II is present only on antigen presenting cells such as DCs, macrophages and B cells. Antigen processing via MHC-I occurs via the proteasome and are presented to CD8+ T cells. In contrast presentation by MHC-II

occurs in the endosomes. MHC-II associated with the invariant chain are targeted to the endosomes. Low pH, cathepsins and human leukocyte antigen (HLA)-DM facilitate antigen loading on MHC-II that can be presented to CD4+ T cells. Antigen encounter results in the maturation of DCs that is characterized by high antigen presentation capacity and lower endocytic activity. DCs in the mature state are characterized by enhanced expression of MHCs, CD80, CD86, and CD40 migrates to regional lymph nodes to initiate antigen specific T cell response. Additionally, in physiological steady state conditions, DCs continuously sample and present self-antigens to CD4+ and CD8+ T cells to maintain peripheral tolerance by deletion or silencing of reactive T cells and expansion of regulatory T cells.

1.4 Adaptive immune system

The adaptive immune system is composed of T and B cells. T cell development occurs in the thymus following migration of lymphoid progenitors from the bone marrow. Commitment to T cell development is marked by the expression of T cell receptor (TCR) which occurs during the end of double negative 2 (DN2) stage. The CD3 complex is formed in the DN3 stage, the CD4 and CD8 co-receptors are formed after the DN4 stage (Hayday and Pennington, 2007). The double positive (DP) thymocytes in the medulla are then positively selected by the thymic epithelial cells (TECs). DP thymocytes that interact with MHC-I become CD8 cytotoxic T cells and DPs that interact with MHC-II become CD4 T helper (Th) cells. Further, the positively selected T cells undergo a negative selection in the thymic cortex to ensure central tolerance (Klein et al., 2009). In this process T cells that interact with self-peptides are eliminated by apoptosis. Thus, the T cell development is highly regulated, where only 1-3% of the thymocytes survive the selection process. Most T cells are composed with a αβ-TCR and a small percentage of T cells acquire the γδ-TCR (Ciofani and Zúñiga-Pflücker, 2010). These naïve T cells then migrate to secondary lymphoid tissues where they are activated upon encountering appropriate antigen coupled to MHCs on APCs. However, the central tolerance mechanisms is not fool proof as some self-reactive T cells escape to the periphery, which upon activation can promote autoimmune diseases. For this reason, ‘peripheral tolerance’ mechanisms mediated by innate and adaptive immune cells are in place to ensure protection.

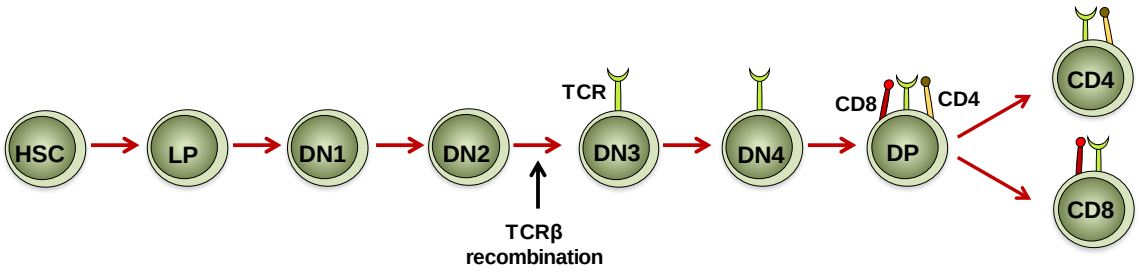

Figure 3. Stages of T cell development

CD4 and CD8 T cells are formed from haematopoietic stem cell (HSC) involving several sequential stages. Lymphoid progenitors (LP), Double negative (DN), Double positive (DP)

1.4.1 Activation of CD4+ T cells

Generation of cellular and humoral responses is governed by the activation and clonal expansion of antigen-specific T helper (Th) cells. This process forms the central event of adaptive immune responses. Activation of naïve Th cells is initiated when the TCR on T cells engages with antigen peptide-loaded MHC-II on the surface of the APC. The TCR-MHC-II engagement forms the first signal (Fig 3), however, this signal alone is insufficient and in the absence of costimulatory signal can lead to anergy.

Further signals in the form of accessory molecules and adhesion molecules are crucial for optimum T cell activation. The interaction between the accessory membrane molecules on APCs such as CD80/86, CD40 with CD28 and CD40L on Th cells is necessary for complete activation and survival of Th cells. This accessory signal represents the second signal which can be a positive or a negative signal. In addition to the costimulatory signal, adhesion molecules on APCs such as CD54 and CD58 engage with CD2 and CD11a on T cells resulting in the clustering of APCs and Th cells. This engagement increases the T-cell-APC interaction at low antigen densities. Finally, differentiation of Th cells into distinct subsets requires a third signal mediated by cytokines derived from activated APCs (Fig 3).

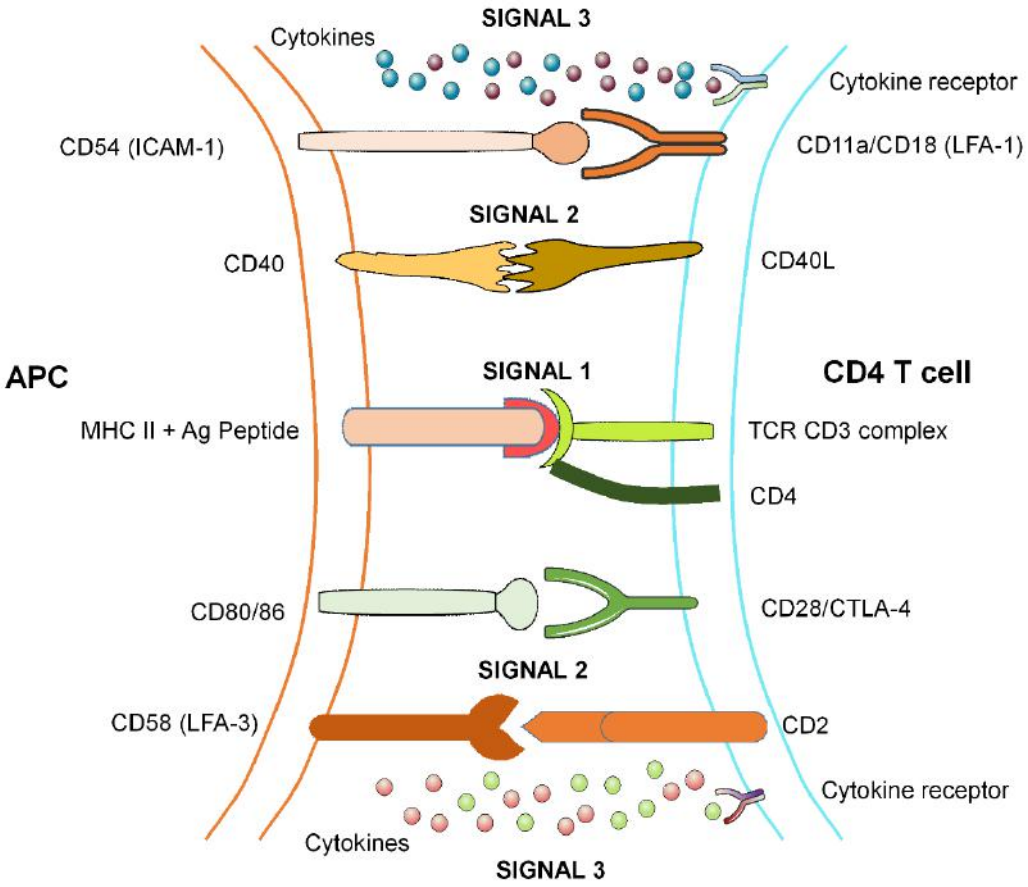

Figure 4. Signals involved in the differentiation of CD4 T cell

Activation of CD4 T cells by antigen presenting cells (APC) involves the interaction of signalling molecules at the APC – CD4 T cell interface. "Antigen educated" APC present antigenic peptides in the context of MHC II to the TCR of naïve CD4 T cells (Signal 1). Co-stimulatory (CD80, CD86, and CD40) and adhesion molecules (CD54 and CD58) interact with their specific ligands on T cells (Signal 2). Cytokines secreted by activated APCs such as IL-6, IL-1β, IL-10, IL-12, and IL-23 (Signal 3) play an essential role in the differentiation of CD4 T cells.

1.4.2 CD4+ T cell differentiation

Differentiation of naïve CD4+ T cells is predominantly governed by the cytokine milieu and is in part aided by the strength of TCR-antigen interaction (Boyton and Altmann, 2002). However, cytokines alone are sufficients for the expansion of Th and subsets does not require further TCR stimulation (Geginat et al., 2001). TCR stimulation enhances IL-2 production from T cells, which signals in an autocrine fashion inducing various cytokine receptors.

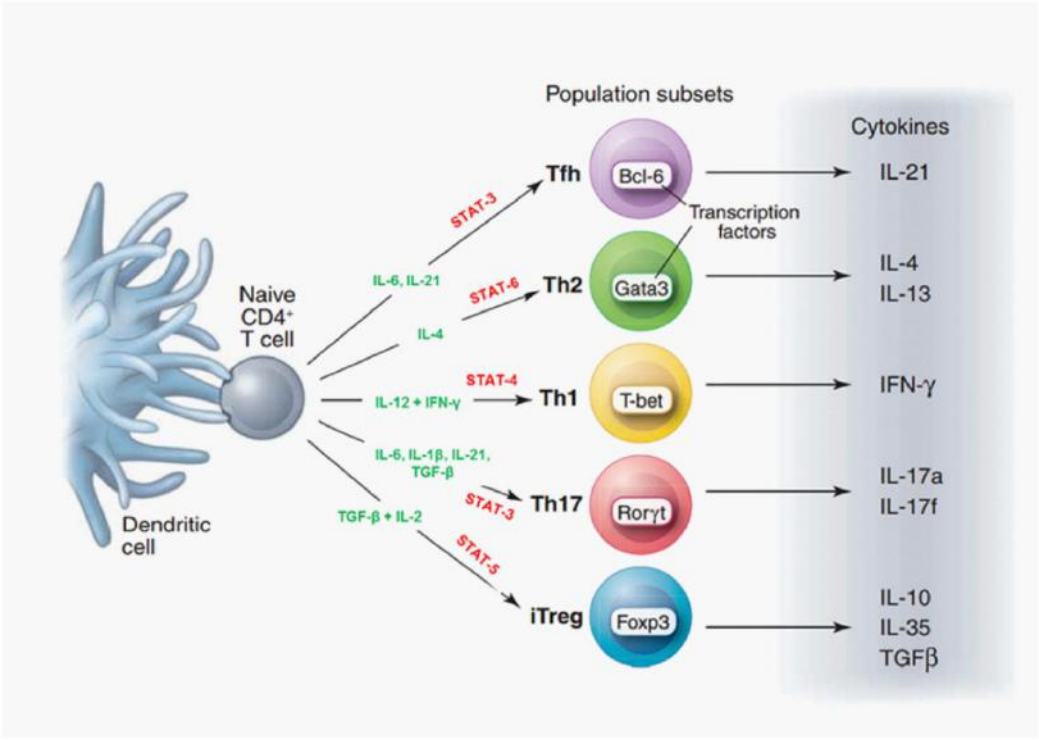

Figure 5. Differentiation of CD4 T cells.

Antigen presenting cells (APCs) such as dendritic cells (DCs) interact with naïve CD4 T cells leading to the differentiation of distinct Th subsets that is dictated by the cytokine milieu in the local environment. Specific set of cytokines promote the differentiation process for each lineage: IL12/IFN-γ for Th1; IL-4 for Th2; TGF-β, IL-6, IL-21, IL-1β for Th17; TGF-β/IL-2 for iTregs; and IL-6, IL-21 for Tfh. Each Th cell lineage express distinct transcription factor and cytokines which are critical for their effector functions. (Figure adapted and modified from O'Shea and Paul, 2010; Science)

Distinct set of innate cytokines produced by activated APCs can now signal and drive lineage-specific transcription factor that culminate in the differentiation of distinct Th subset. The initial paradigm of Th1 and Th2 that was first described by Mossman and Coffman in 1986 has now expanded and includes Th17, T follicular helpher (Tfh) and Tregs as additional Th subsets (Mosmann et al., 1986; Mosmann and Coffman, 1989; O'Shea and Paul, 2010). IL-12/IFN-γ drives Th1 differentiation; IL-4/(IL-2) for Th2; IL-6, IL-1 β, IL-23, IL-21 and TGF-β for Th17; TGF-β and retinoic acid (RA) for Tregs; and IL-21 and IL-6 for Tfh (Fig 4).

In addition to the aforementioned Th subsets, other potential Th subsets have been described. Th9 and Th22 cells are an effector subset which can secrete IL-9 and IL-22 respectively (Dardalhon et al., 2008; Eyerich et al., 2009; Schmitt and Ueno, 2015). Th3 (TGF-β-secreting CD4 T cells) (Chen et al., 1994; Weiner, 2001), Tr1 (IL-10-secreting CD4 T cells)

(Groux et al., 1997), Tr35 (IL-35-secreting CD4 T cells), (Collison et al., 2010; Vignali and Kuchroo, 2012) and Tfr cells (Sage and Sharpe, 2016). Tfr cells have been implicated in inhibiting the functions of Tfh cells (Miles et al., 2015).

1.4.2.1 Th1 differentiation

The initial TCR engagement activates Tbx21 genes and induces low levels of IFN-γ. IL-12 (Amsen et al., 2009). The pivotal Th1 differentiation cytokine produced by activated APCs, signals through IL-12R inducing IFN-γ in a STAT-4-dependent pathway. IFN-γ produced by autocrine/paracrine signals bind to the IFN-γR inducing STAT-1 expression (Figure 5). STAT-1 strongly induces the expression of Tbet the master regulator of Th1 differentiation and alternatively inhibits Treg development by suppressing FOXP3 expression (Caretto et al., 2010). Tbet orchestrates Th1 differentiation by activating Th1 signature genes which are achieved by recruitment of chromatin remodelling complexes to the target site (Miller and Weinmann, 2010). Additionally, Tbet represses alternative lineage development by directly or indirectly preventing the functions of other lineage master regulators. Tbet directly sequesters GATA3 and inhibits Th2 differentiation (Hwang et al., 2005; Oestreich and Weinmann, 2012). Further, Tbet binds to Runx1 inhibiting Th17 differentiation by preventing Runx1-dependent RORC expression (Djuretic et al., 2007; Lazarevic et al., 2011).

The principle cytokines secreted by Th1 cells are IFN-γ, TNF-α, lymphotoxin-α, and IL-2. Th1 response is associated with cell mediated immunity and plays a dominant role in the elimination of intracellular pathogens. IFN-γ is a critical cytokine that enhances microbicidal and tumoricidal activities (Ikeda et al., 2002; O'Garra and Robinson, 2004). Although, Th1 response play a fundamental role in the clearance of pathogens and tumors, the proinflammatory properties of Th1 cells can also cause tissue damage and provoke autoimmune diseases (Pakala et al., 1999; Wang et al., 1997). They also exacerbate inflammatory diseases and play a detrimental role in graft-versus-host disease (Davidson et al., 1996; Hu et al., 1999; Parronchi et al., 1997).

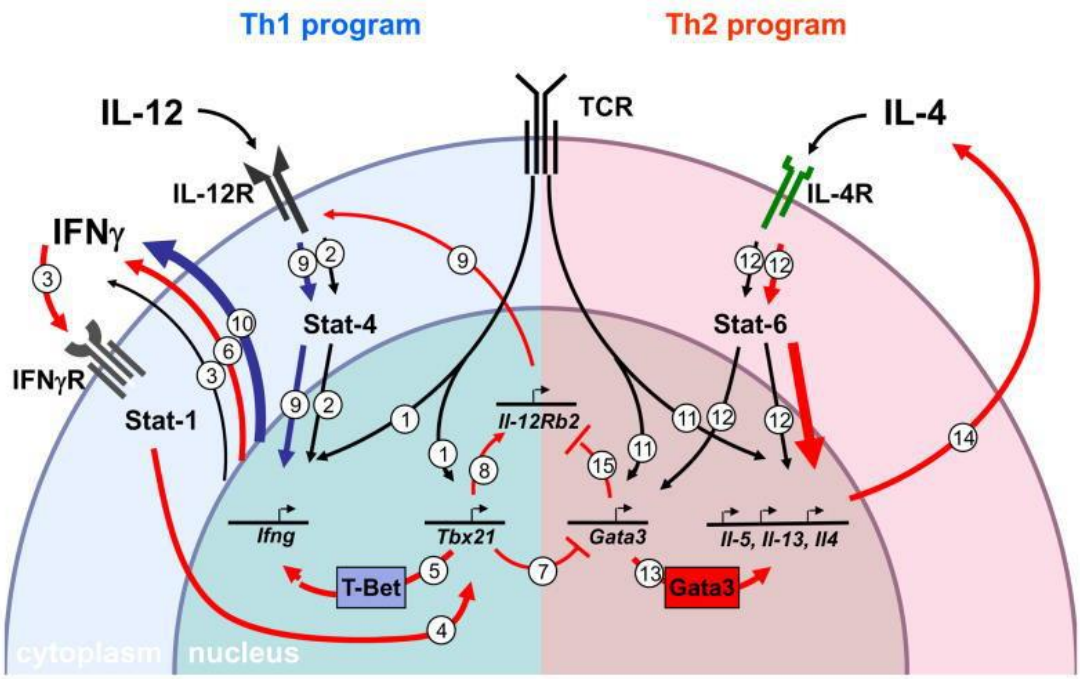

Figure 6. IL12 and IL4 driven T helper differentiation

Th1 induction by IL12: Initial TCR activation induces low grade expression of the *Ifng* and the *Tbx21* genes (1). Signaling through the IL12 receptor results in STAT4 mediated promotion of IFNγ expression (2). Binding of the IFNγ receptor by low initial auto/paracrine produced IFNγ activates STAT1 (3), which strongly promotes expression of the Tbx21 gene (4). T-bet then enhances the transcriptional competence of the *Ifng* gene (5) leading to increased production of this cytokine (6). In addition, T-bet prevents Th2 differentiation by inhibiting Gata3 (7). Finally, T-bet promotes expression of the IL12 receptor β2 chain (8), resulting in greater IL12 responsiveness (9) and yet further elevated production of IFNγ (10).

Th2 induction by IL4: Initial TCR signaling induces low level expression of the *Il4* and *Gata3* genes (11). IL4 receptor signaling strongly promotes expression of these two genes (12). Gata3 reorganizes chromatin structure in the Th2 locus, encompassing the *Il4*, *Il5* and *Il13* genes, enhancing their transcription competence (13). Increased IL4 production further enhances TH2-cell differentiation in a fed forward loop (14). Finally, Gata3 prevents the Th1 differentiation program by inhibiting expression of the IL12 receptor β2 chain (14) and of the *Stat4* gene (not depicted). Primary events are indicated with black arrows, secondary events with red arrows and tertiary events with blue arrows. (Adapted from Amsen et al., 2009; Curr Opin Immunol).

1.4.2.2 Th2 differentiation

Th2 differentiation necessities the signalling via IL-4. Initial TCR signalling induces low levels of IL-4 and GATA3, akin to Th1 differentiation. IL-4 binds to its cognate receptor IL-4R and activates STAT-6 which subsequently induces the expression of GATA3, the master

regulator of Th2 differentiation (Amsen et al., 2009) (Figure 5). Further, growth factor independent-1 (Gfi-1), a transcriptional repressor induced by the IL-4/STAT-6 signalling favours Th2 development by selecting GATA3 high cells (Zhu et al., 2002). GATA3 orchestrates Th2 differentiation by remodelling the chromatin structure in the Th2 locus, which encompasses *il4*, *il5*, and *il13* genes. Importantly, GATA3 represses Th1 development by antagonizing the functions of Tbet and by curtailing the IL-12/STAT-4 axis involved in the accentuation of Th1 development (Zhu et al., 2006). Although GATA3 ensures the development of Th2 cells, additional signalling mediated by IL-2/STAT-5 to stabilize the IL-4 locus and IL-4Rα is necessary in achieving a prominent Th2 response (Liao et al., 2008; Zhu et al., 2003). Exogenous IL-4 derived from basophils, mast cells, and NKT cells further escalate Th2 differentiation in a feed forward loop.

The principle cytokines secreted by Th2 cells are IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin. Unlike Th1 response, Th2 response is generally associated with humoral immune response. Th2 response directs the generation of pathogen-specific immunoglobulins which neutralizes invading pathogens. Therefore Th2 response is important in the host defense against extracellular parasites such as helminths and nematodes (Anthony et al., 2007). Th2 cells also play an important role in suppressing Th1-driven autoimmune disease, neutralization of toxins, and wound repair. However, Th2 response orchestrates a complex inflammatory response that is involved in the induction and persistence of several allergies and asthma (Licona-Limón et al., 2013; Steinke and Borish, 2001).

1.4.2.3 Th17 differentiation

The Th1/Th2 paradigm was expanded to include a third effector subset termed Th17 due to the production of IL-17 as their signature cytokine. Similar to Th1 and Th2 cells, differentiation factors and lineage specific transcription factor that is unique to Th17 cells orchestrate Th17 differentiation. Studies in mice indicated that the combination of cytokines TGF-β and IL-6 was necessary for the differentiation of Th17 cells from naïve Th cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). Although, TGF-β and IL-6 had

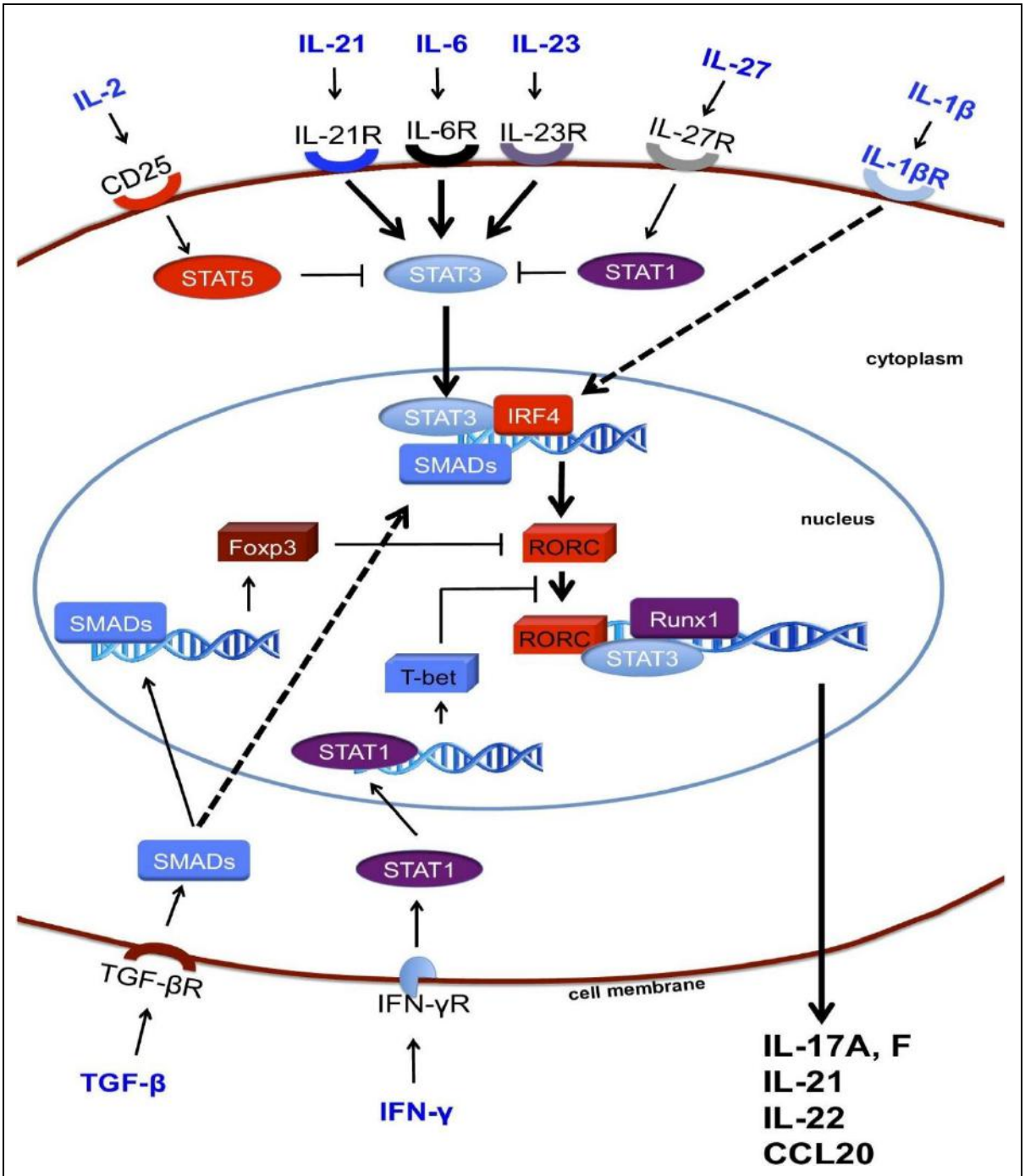

Figure 7. Th17 differentiation.

TGF-β is essential for the generation of both induced regulatory T cells and Th17 cells via the induction of FoxP3 and RORC. However, in the absence of inflammation, FoxP3 represses RORC and promotes iTregs. Signaling via inflammatory cytokines, such as IL-6, IL-21, and IL-23, results in STAT3 phosphorylation, relieves RORC from the suppression of FoxP3, and initiates Th17 programming. STAT3 in combination with IFN regulatory factor 4 (IRF4) further induces RORC expression. The transcription factors STAT3, RORC, and Runx1 bind to the promoter regions of the IL17, IL21, IL22, and CCL20 genes and induce IL-17, IL-21, IL-22, and CCL20. Th17 programming can be antagonized by cytokines, such as IFN-γ, IL-2, and IL-27. IL-2-mediated and IL-27-mediated activation of STAT5 and STAT1 inhibit STAT3, whereas T-bet induced by IFN-γ can block RORC. (Adapted from Maddur et al., 2012)

opposing functions, in combination they were capable of inducing the lineage-specific regulator retinoic acid-related orphan nuclear receptor (ROR-γt), suggesting that the downstream signalling molecules and transcription factors need to cooperate in driving Th17 differentiation (Bettelli et al., 2006; Dong, 2008; Mangan et al., 2006). Interestingly, IL-6$^{-/-}$ mice were capable of generating Th17 response, which led to the identification that IL-21 along with TGF-β can orchestrate an alternative Th17 differentiation in naïve T cells (Korn et al., 2007). As Th17 cells themselves express high levels of TGF-β and are the major source of IL-21, an autocrine amplification loop has been proposed (Korn et al., 2009, 2007; Nurieva et al., 2007; Zhou et al., 2007).

Translation of the Th17 differentiation knowledge from mice to humans initially met with discrepancies with regard to the cytokine cocktail required for Th17 differentiation. In particular, the role of TGF-β in human Th17 differentiation was questioned. It was proposed that TGF-β was dispensable for the differentiation of human Th17 differentiation from naïve T cells. Th17 differentiation can be achieved by a combination of IL-1β and IL-6/IL-23 or IL-23 alone (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). However, these results were biased due to the fact that, differentiation of Th17 cells was performed using CD45RA+ Th cells from peripheral blood. Furthermore, in these reports the endogenous source of TGF-β (eg, serum and platelets) was not carefully controlled. Subsequent studies done with rigorously sorted naïve T cells and performing the differentiation assay in serum-free medium suggested that, TGF-β is indispensable for the differentiation of human Th17 cells from naïve T cells (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008). Importantly, it was shown that Th17 differentiation can be achieved by using just TGF-β and IL-21, whereas IL-1β and IL-6 was important to expand the differentiated Th17 cells (Yang et al., 2008). Mechanistically, TGF-β and IL-6/IL-21 induces the expression of IL-1R and IL-23R, making the cells receptive to IL-1β and IL-23 (Maddur et al., 2012; Manel et al., 2008; Yang et al., 2008). Further, TGF-β can also activate RORC and inhibit Th1 response by suppressing Tbet (Santarlasci et al., 2009) (Fig 6).

1.4.2.4 Regulatory T cells

In the early1970s, several groups demonstrated that thymic derived T cells in addition to augmenting immune response can also repress immune response (Dorf and Benacerraf, 1984; Gershon and Kondo, 1970; Green et al., 1983; Ohki et al., 1987). However a reliable marker to work with these suppressor cells was absent until the identification of CD25 which

was shown to be preferentially expressed on a subset of T cells (Sakaguchi et al., 1995). It is now clear that these suppressor cells termed as "Tregs" form a subset of T cells that are developmentally and functionally distinct. Tregs can either be developed in the thymus (nTregs or tTregs) or induced in the periphery (iTregs). Development of nTregs occurs in the thymus under the instruction of thymic APCs (cortical and medullary TECs and thymic DCs). TCR-mediated self-antigen recognition, co-stimulatory signals, TGF-β signaling, and importantly, engagement of the common gamma chain cytokine receptor IL-2R facilitates the differentiation of nTregs (Malek et al., 2008; Picca et al., 2006). Interestingly, unlike conventional T cells that can produce IL-2, Tregs do not produce IL-2 due to chromatin inaccessibility of the IL-2 locus (Su et al., 2004). Consequently, survival and growth of Tregs is dependent on paracrine IL-2. TCR engagement with the MHC-II: self-peptide induces IL-2Rα (CD25) and thus precursor cells become receptive to IL-2. Further TCR and costimulatory signaling induced NFAT and cRel, Creb, along with IL-2 induced STAT-5 are recruited to the FOXP3 promoter to initiate FOXP3 transcription, the lineage marker for Tregs (Kim and Leonard, 2007; Littman and Rudensky, 2010; Long et al., 2009; Zheng et al., 2010). Owing to their central role in controlling self-reactive T cells, nTregs prevent autoimmunity, limit immunopathology, and maintain immune homeostasis.

Adaptive Tregs or iTregs develop outside the thymus in a tolerogenic environment. iTregs are differentiated from naïve CD4 T cells upon immunogenic or subimmunogenic antigen presentation during chronic inflammation, infection, and during normal homeostasis in the gut. Differentiation of iTregs requires the cytokines TGF-β and IL-2. Importantly, due to plasticity of T cells FOXP3 expression can be lost, resulting in loss of suppressive activity or gain of effector functions (Bailey-Bucktrout et al., 2013; Hori, 2014; Zhou et al., 2009). Genome wide analysis of Tregs and conventional T cells has indicated that Tregs have differential methylation status throughout the genome (Ohkura et al., 2012; Zhang et al., 2013). Notably the Treg-cell-specific regions such as the FOXP3 intron1 which encompasses the genes FOXP3, *Ikfz4*, and CTLA-4 (exon 2) needs to be hypomethylated for stable expression of FOXP3 and for maximal suppressive functions of Tregs. TGF-β signaling can alter histone modification status of the FOXP3 region, which assembles transcription factors and transactivate Foxp3 expression (Hill et al., 2007; Ogawa et al., 2014; Ohkura et al., 2013, 2012). Additionally, signaling mediated by programmed death-1 - programmed death-1-ligand-1, CTLA-4, OX40-OX40L can augment the generation of iTregs.

Tregs are indispensable in the maintenance of immunological self-tolerance and homeostasis. Dysregulation of Tregs is associated with a number of autoimmune diseases, allergies and immunopathology. They are actively involved in suppressing the activation of effector functions of diverse immune cells, such as CD4+ and CD8+ T cells, DCs, B cells, monocytes, macrophages, mast cells, NK cells, and NKT cells (André et al., 2009; Shevach, 2006; Tang and Bluestone, 2008).

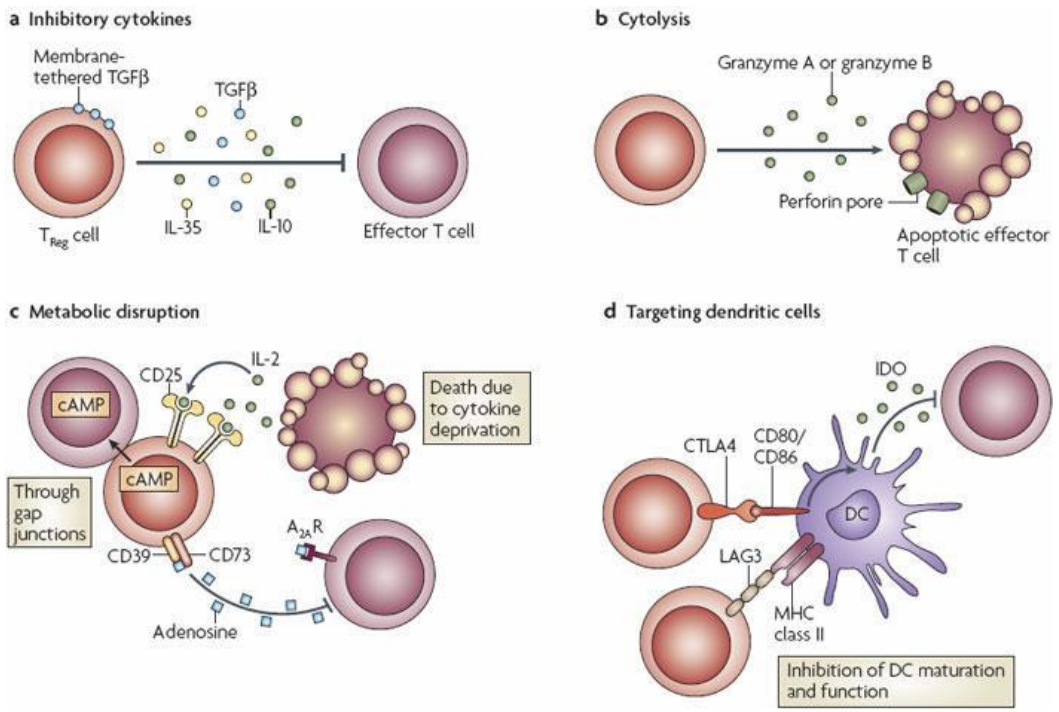

Figure 8. Treg mediated suppressive mechanism

(a) Inhibitory cytokines secreted by Tregs such as IL-10, IL-35, and TGF-β supress effector T cell functions. (b) Granzymes and perforins secreted by Tregs effectuate cytolysis by direct killing. (c) Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor α)-dependent cytokine deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39/CD73 – generated, adenosine receptor 2A (A2AR)-mediated immunosuppression. (d) Targeting dendritic cells (DCs) includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3)–MHC II mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs. (Adapted from Vignali et al., 2008; Nat Rev Immunol)

Suppressive functions of Tregs can be mediated by contact dependent or independent mechanisms (Vignali et al., 2008). Suppressive cytokines and cell death inducing proteins secreted by Tregs mediate contact independent suppressive mechanisms. Inhibitory cytokines such as IL-10, TGF-β, IL-35 and fibrinogen-like protein 2 (FGL2) are key mediators involved in repressing effector T cell functions (Shalev et al., 2011; Vignali et al., 2008). Additionally,

granzymes and perforins effectuate cytolysis by direct cell killing (Grossman et al., 2004). The ectoenzymes CD39 and CD73 and inhibitory receptors on Tregs mediate contact-dependent suppressive functions. CD39 and CD73-induced adenosine converts T cells into iTregs. cyclic AMP causes metabolic disruption in conventional T cells (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006) (Figure 7). Lastly, the panoply of inhibitory receptors on Tregs suppresses dendritic cell functions. CTLA-4 and LAG-3 on Tregs are implicated in suppressing DC functions. Tregs constitutively express CTLA-4, which upon interaction with CD80/86 on DCs confers regulatory features in DCs. Treg-educated DCs are involved in attenuating activation of effector T cells. Further, Tregs can program DCs to secrete indoleamine 2,3-dioxygenase (IDO) which is a potent regulatory molecule that can supress effector T cell response (Fallarino et al., 2003; Mellor and Munn, 2004). Further, interaction of LAG-3 with MHC-II inhibits DC maturation (Huang et al., 2004).

2 Aspergillus fumigatus

2.1 Clinical relevance of fungal pathogens

Of the diverse classes of pathogens that cause human diseases, fungal pathogens are the least characterized and often underappreciated. One of the reasons is that healthy individuals largely do not get fungi-mediated life-threatening diseases. However, considering that fungal infections cause ~1.3 million deaths annually (Rutgers biomedical and health sciences, Brown et al., 2012)), which is similar to the number of deaths caused by *M. tuberculosis* infections, an effort to characterize fungal pathogens is necessary. Fungal pathogens cause a wide range of diseases ranging from superficial infections, allergies and life-threatening invasive diseases. Superficial fungal infections occur in ~25% of the world's population, however, most of them can be easily treated (Brown et al., 2012). In contrast, although the incidence of invasive fungal disease is relatively lesser than superficial infections, it is of great concern due to the high mortality rate. Majority of the invasive fungal disease is caused by species that belong to one of the four genera: *A. fumigatus*, *Candida*, *Cryptococcus and Pneumocystis.*

2.2 *A. fumigatus* associated diseases

A. fumigatus is a ubiquitously distributed, largely saprophytic, filamentous fungi that belong to the phylum Ascomycota. It plays an essential role in the carbon - nitrogen fixation (Millner et al., 1977). More than 200 species of *A. fumigatus* has been documented and most of which are non-pathogenic with some of the species, even being used in the industrial production of enzymes and fermented products (Østergaard and Olsen, 2011). Relatively very few species of aspergillus are pathogenic among which *A. fumigatus* is the principal causative agent of most respiratory infections in humans. *A. fumigatus* causes a spectrum of diseases depending on the immune status of the host (Figure 8). In individuals with altered lung functions such as cystic fibrosis and asthma, *A. fumigatus* can cause a hypersensitive response to fungal components, termed allergic bronchopulmonary aspergillosis (ABPA).

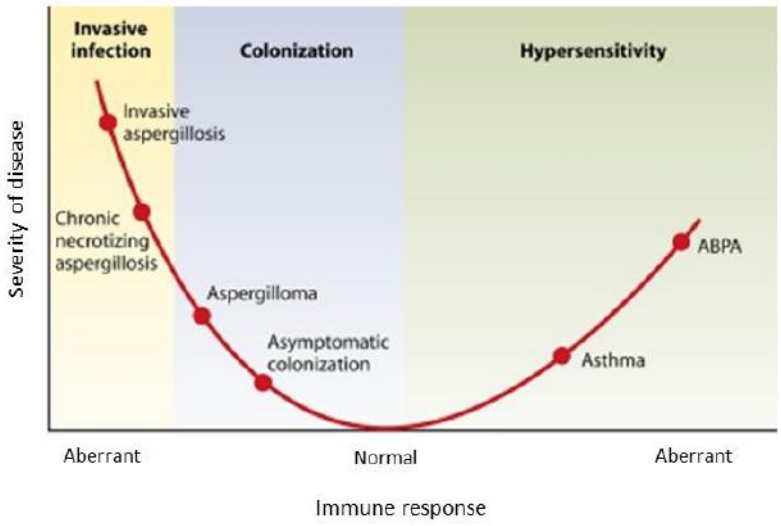

Figure 9. Diseases attributable to *A. fumigatus* as a function of the host's immune status.

The range of disease caused by *A. fumigatus* in humans. Severely immunocompromised individuals are susceptible in developing invasive aspergillosis. Individuals with mild immunosuppression or chronic lung disease may develop chronic necrotizing aspergillosis. Pre-existing cavities in the lungs form a niche for the development of aspergilloma. Constant exposure to *A. fumigatus* may lead to the development of asthma. Individuals with asthma and cystic fibrosis are highly susceptible in developing allergic bronchopulmonary aspergillosis (ABPA) (Figure adapted and modified from Park SJ, Mehrad B)

Preexisting cavities in the lungs such as the healed lesions in tuberculosis patients become a site for non-invasive aspergillomas, a persistent colonization and the formation of hyphal mass due to repeated exposure to conidia. Invasive Aspergillosis (IA) (Park and Mehrad, 2009), the life threatening disease caused by *A. fumigatus* occurs in

immunocompromised individuals. The risk cohort for IA includes individuals with prolonged neutropenia, genetic immunodeficiency such as chronic granulomatous disease (CGD), HIV/AIDS patients, individuals with hematological malignancies such as leukemia, patients undergoing solid organ or haematopoietic stem cell transplantation, and patients receiving chemotherapy or prolonged corticosteroid therapy (Brown and Netea, 2012; Cornillet et al., 2006; Latgé, 1999; Park et al., 2009). IA usually leads to pneumonia and if not treated, can spread rapidly to different organs, leading to death of the patient.

Management of IA is complicated due to rapid progression of the disease which becomes very difficult to treat, as a result mortality rate is high which can range from 30-95% (Brown et al., 2012). Although, significant efforts in the past decades have uncovered a wealth of information about our understanding of host-fungal interactions, treatment outcome for IA remains poor with a very high mortality rate. Additionally, the emergence of azole resistance strains complicates the already growing problem in managing IA (Vermeulen et al., 2013). Therefore, deciphering the immune response to *A. fumigatus* and in parallel identifying new anti-fungal drugs will be critical in developing novel therapies in combating IA.

2.3 Pathogenesis of *A. fumigatus*

A. fumigatus mainly reproduces by asexual means which results in the production of large number of spores called conidia. The conidia can be easily dispersed in the air and in a suitable environment the dormant conidia can swell and germinate to form the mycelium. Hundreds of airborne conidia are inhaled by humans on a daily basis (Latgé, 1999). Due to the small diameter of the conidia (2 to 3um), they can easily reach the human alveoli, the origin for most systematic *A. fumigatus* infection. The alveoli comprise of type I and type II airway epithelial cells (AEC) (Herzog et al., 2008). Invasion of epithelial cells is initiated with the adherence of conidia to type II AEC (McCormick et al., 2010b). Conidial proteins such as fucose-specific lectin A and thaumatin domain protein AfCalAp have been implicated in the adhesion process (Houser et al., 2013; Upadhyay et al., 2009). Further, conidia can bind to basement membrane proteins composed of several matrix proteins such as fibrinogen, laminin, and type I and type IV collagen (Bromley and Donaldson, 1996). Also the conidial surface has been shown to contain sialic acids which are implicated in the binding process (Warwas et al., 2007; Wasylnka et al., 2001). Adherence of conidia to type II AECs triggers the formation of pseudopods leading to engulfment of the conidia. Once the conidia are internalized, they are trafficked through the endosomes which fuse with the lysosomes forming the phagolysosome

(Botterel et al., 2008; Wasylnka and Moore, 2003). Most of the conidia are killed within the acidic compartment of the phagolysosome, however, some of them can survive and germinate. The resulting hyphae can penetrate the plasma membrane of type II AEC without causing any detectable damage to the host (Filler and Sheppard, 2006). Interestingly, as seen in some intracellular pathogens such as *M. tuberculosis*, the conidia actively inhibits apoptosis of type II AECs (Berkova et al., 2006), which may contribute to the pathogenesis of *A. fumigatus*. Finally, the hyphae invade the blood vessels and disseminate to other organs culminating in active invasive disease.

2.4 Immune response to *A. fumigatus*

Several components of the immune system are involved in mediating host response to inhaled conidia (Figure 9). *A. fumigatus* conidia that enters the respiratory tract are largely eliminated by the airway mucosa and epithelia. The pulmonary fluid lining the respiratory tract contains mucus, proteins, ions, lipids, and cellular secretions, which aid in the mucociliary clearance of inhaled conidia. Additionally, the pulmonary fluid consists of soluble opsonic PRRs, which contribute to host defense. Type II AECs and clara cells secrete various collectins which include mannose binding lectin (MBL), and the surfactant proteins SP-A and SP-D (Kishor et al., 2002; Singh et al., 2015). Collectins are a group of C-type lectin receptors that function as opsonins by binding to carbohydrate moieties present on pathogens in a calcium-dependent manner. *In vitro* studies have shown that SP-A, and SP-D can agglutinate *A. fumigatus* conidia and enhance their phagocytosis and killing by macrophages and neutrophils (Brummer and Stevens, 2010; Madan et al., 1997). Further, SP-A and SP-D have been demonstrated to play an important role *in vivo* as SP-$A^{-/-}$ and SP-$D^{-/-}$ mice exhibit enhanced susceptibility to IA (Madan et al., 2005).

Complements, a collection of serum proteins predominantly found in the serum are also present in the bronchoalveolar fluid albeit at lower concentrations in comparison to serum. Complements are shown to opsonize pathogens and enhance phagocytosis. Different morphotypes of *A. fumigatus* has been shown to activate the complement cascade which results in the deposition of C3 on the surface of *A. fumigatus* (Kozel et al., 1989). Upon binding the C3 complement is cleaved to iC3b, a ligand for complement receptor C3R on phagocytic cells (J. Sturtevant and Latgé, 1992; J. E. Sturtevant and Latgé, 1992). Further, mannan-binding lectin (MBL) promotes the activation of lectin complement pathway via C4bC2a, resulting in

the deposition of complements on the surface of conidia and hyphae (Kaur et al., 2007). Additionally, *A. fumigatus* conidia when cultured in healthy human serum activated the

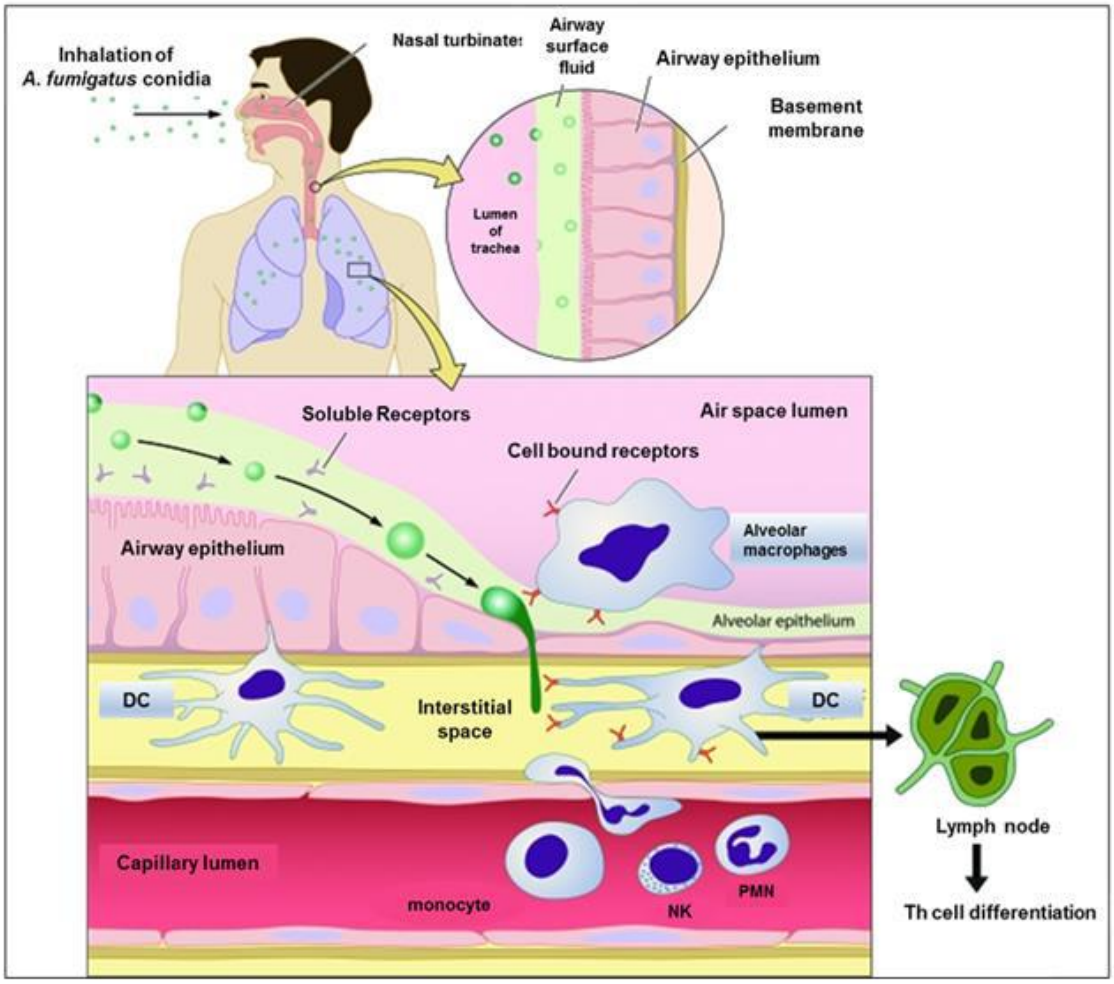

Figure 10. Components of host response to inhaled *A. fumigatus* conidia.

First line of defense against inhaled Aspergillus conidia is constituted by the mucociliary clearance aided by the pulmonary fluid of the airway epithelium. Soluble and cell bound receptors contribute to phagocytosis of the conidia by alveolar macrophages. Immune cells (monocytes, NK cells, PMNs, and DCs) recruited to the site of infection contribute to innate anti-fungal defense mechanisms. *Aspergillus*-derived PAMPs induces the activation of DCs which migrate to the draining lymph nodes to prime antigen specific Th cell response. NK, Natural killer PMN, polymorphonuclear leukocyte (neutrophil); Th, T helper. (Figure

alternative complement pathway via the MBL C2 bypass mechanism (Dumestre-Pérard et al., 2008). Importantly, clinical isolates of *A. fumigatus* isolated from patients with invasive aspergillosis induced a stronger activation of the alternative complement pathway as compared to the environmental isolates (Dumestre-Pérard et al., 2008). Thus, the activation of complement pathway may be influenced by antigenic structures on *A. fumigatus*. In addition to collectins and complements, long chain pentraxin 3 (PTX3) is an important soluble receptor

involved in the defense against *A. fumigatus*. Inflammatory mediators can stimulate the secretion of PTX3 from phagocytes and DCs. PTX3 functions as an opsonin and can also activate complement through the classical pathway by binding to C1q and ficolin-2 (Ma et al., 2009). Mice deficient of PTX3 have reduced recognition of *A. fumigatus* and are susceptible to IA (Garlanda et al., 2002).

2.4.1 Interaction of *A. fumigatus* with epithelial cells

Epithelial cells play an important role in the early phase of the immune response to *A. fumigatus* infection. Epithelial cells are among the first cells that come in direct contact with inhaled conidia. Upon pathogen encounter, epithelial cells can secrete antimicrobial peptides a group of highly conserved proteins which have broad-spectrum activity against invading pathogens. *A. fumigatus* has been shown to induce the secretion of human β-defensins hBD2 and hBD9 from respiratory epithelial cells (Alekseeva et al., 2009; Sun et al., 2012). Type II epithelial cells are also involved in the clearance of the conidia by internalizing conidia and killing via phagocytosis. Additionally, epithelial cells can induce the production of cytokines and chemokines such as IL-6, TNF-α, and IL-8 thereby assisting in the initiation of inflammation and in recruitment of neutrophils (Balloy et al., 2008; Bellanger et al., 2009).

2.4.2 Interaction of *A. fumigatus* with innate cells

Alveolar macrophages (AM) are among the first immune cells that interact with respiratory pathogens. Inhaled conidia are phagocytosed and trafficked to phagolysosome for killing (Ibrahim-Granet et al., 2003). Further, swollen conidia are sensed by PRRs on AMs triggering ROS production via the p47phox-NADPH oxidase complex. Dectin-1 and among TLRs, TLR2 and TLR4 are the major PRRs implicated in sensing *A. fumigatus* PAMPs (Brown et al., 2003; Figueiredo et al., 2011; van de Veerdonk et al., 2008). PRR engagement with *A. fumigatus*-derived PAMPs such as β-(1-3)-glucan induces the production of cytokines and chemokines that mediate host defense. TNF-α, IL-1β, and IL-6 drive inflammatory response, IL-8 and macrophage inflammatory protein-2 (MIP-2) and involved in the recruitment of neutrophils to the site of infection (Morrison et al., 2003; Phadke and Mehrad, 2005).

Recruited neutrophils play an indispensable role in the control of *A. fumigatus* infection. Indeed, neutropenia is a major risk factor for developing IA. Phagocytosis and NADPH oxidase-mediated ROS production is the major mechanism of neutrophil-mediated fungicidal activity (Bonnett et al., 2006; Grimm et al., 2011; Morton et al., 2012). Additionally,

neutrophils secrete azurophil granules which are loaded with hydrolytic enzymes such as myeloperoxidase, cathepsin G, elastase, and proteinase 3 which are implicated in hyphal degradation (Morton et al., 2012; Segal, 2005; Spitznagel, 1990). NETosis triggered by the oxidative burst results in the formation of NETs which are involved in curtailing hyphal growth (McCormick et al., 2010a).

Table 3. Effector functions of different cells involved in host defense against *A. fumigatus*

Cell type	Role in host defense against *A. fumigatus*
Epithelial cells	Production of anti-microbial peptides such as β-defensins Killing of inhaled conidia by phagocytosis Production of proinflamatory cytokines
Alveolar macrophages	Phagocytosis and killing of inhaled conidia Initiation of inflammatory response Recruitment of monocytes and neutrophils
Neutrophils	Phagocytosis of sporulating conidia Generation of ROS and Production of lactoferrin Release of antimicrobial proteases by degranulation Formation of NETs
Monocytes	Phagocytosis of conidia Precursors for macrophages and DCs Production of proinflammatory cytokines Shaping of adaptive immune response
Myeloid dendritic cells	Recognition and uptake of conidia and germ tubes for antigen processing Production of proinflammatory and polarizing cytokines Priming antigen-specific Th cells
NK cells	Direct cytotoxic effects against hyphae mediated by perforins and granzymes Production of IFN-γ
pDCs	Production of type 1 interferons Formation of pET
Platelets	Conidial and hyphal killing by release of granules such as serotonin

(Content of this table is summarized in Espinosa V and Rivera A, 2016; Morton CO 2012)

Circulating monocytes which form a reservoir for tissue macrophages and DCs are recruited to the site of infection. Monocytes enhance host defense by phagocytoses of the conidia and in sustaining the inflammatory cytokine milieu in the lungs (Diamond et al., 1983; Espinosa and Rivera, 2016; Roilides et al., 1995., Hohl et al., 2009; Serbina et al., 2009)). CCR2+ Ly6c^{hi} inflammatory monocytes, the human CD14+ monocyte counterpart differentiates into inflammatory DCs (Mo-DC) or macrophages depending on the cytokine signalling they receive. PRR engagement with *A. fumigatus* PAMPs induces maturation and activation of DCs. Activated DCs migrate to the draining lymph nodes to prime antigen-specific T cell response. DCs also secrete various chemokines, which recruit innate and adaptive effector cells to the site of infection. CCL3, CCL4, CXCL10 and CCL20 secreted by DCs in response to *A. fumigatus* mediate the recruitment of effector memory Th1 cells. IFN-γ produced by Th1 cells enhances innate immune cell functions and aids in fungal clearance (Bozza et al., 2002; Espinosa and Rivera, 2016; Gafa et al., 2007).

2.5 Cell wall of *A. fumigatus*

The fungal cell is surrounded by a cell wall which plays an essential role in the growth, survival, and morphogenesis of the fungi. The *A. fumigatus* cell wall acts as a sieve and reservoir for effector molecules such as antigens and enzymes. The cell wall is predominantly composed of different polysaccharides which can be fractionated into alkali soluble and

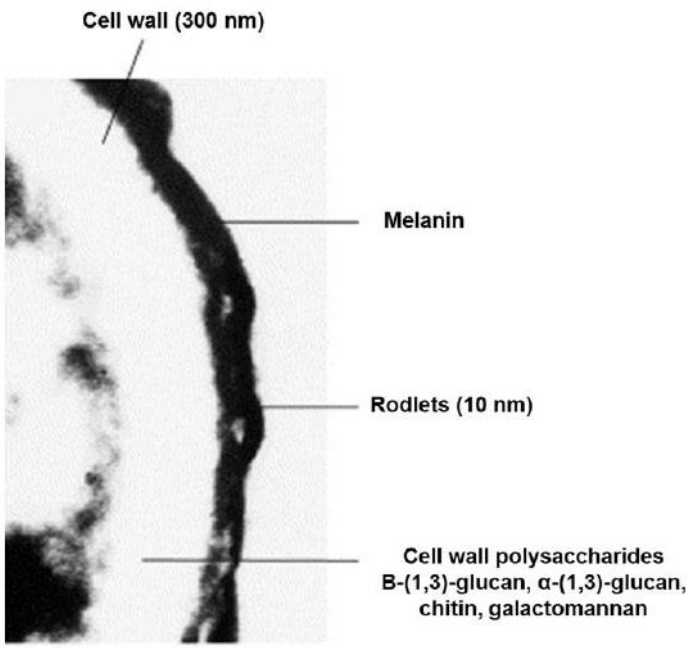

Figure 11. Structural organization A. fumigatus cell wall.

Electron microscopy section of the conidial cell wall depicting the translucent inner layer which is composed of structural polysaccharides and proteins whereas the electron-dense outer layer contains melanin. The outer layer consists of regularly arranged hydrophobic rodlet proteins which form the hydrophobin layer. (Figure adapted and modified from Latge JP, 2001)

insoluble fractions. The fibrillar core consisting of a mixture of branched β-(1-3)-glucan and (4%) β-(1-6)-glucan. The β-glucans are covalently bound by chitin/chitosan, β-(1,3)/β-(1,4)-glucan and β-(1,5) galacto-α-(1-2)/α-(1-6) mannan. The alkali-soluble amorphous fraction is largely constituted of linear chain α-(1-3)-glucans along with intra-chain α-(1-3)/α-(1-4)-glucans, galactomannan and galactosaminogalactan (GAG) (Latgé and Beauvais, 2014). The concentration and localization of *A. fumigatus* cell wall polysaccharides differ between the conidial and hyphal morphotypes (Latgé, 2010). Of note, the conidial cell wall is composed of a superficial hydrophobic rodlet layer made up of RodA protein followed by the melanin layer (Latgé, 2001) (Figure 11). This hydrophobic layer is immunologically inert and masks immune recognition of the conidia (Aimanianda et al., 2009). In contrast, the outer layer of hyphae consists of galactosaminogalactan (GAG), which has been implicated in the pathogenesis of aspergillosis. Conidial germination triggers morphological changes that involves degradation of the proteinaceous hydrophobic and melanin layer, exposing the masked inner cell wall composed of polysaccharides. As the cell wall constituents are fungal-specific, they are recognized by the innate immune system. Noteworthy, the unique constituents of fungal cell wall form an attractive target for drug development (Tada et al., 2013).

2.6 Role of PRRs in *A. fumigatus* recognition

Activation of innate immune system is triggered upon recognition of pathogen via PRRs. Depending on the morphological state of the fungi multiple PRRs in different combination may be involved in the recognition process. The overall immune response depends on the extent of stimulation of individual receptors, cooperativity and cellular localization of receptors. Innate immune cells harbour a plethora of PRRs of which Dectin-1, TLR-2, TLR-4, and DC-specific ICAM3-grabbing non-integrin (DC-SIGN) are the major PRRs implicated in the recognition of *A. fumigatus* (Brown et al., 2003; Figueiredo et al., 2011; van de Veerdonk et al., 2008) (Figure11).

2.6.1 Dectin-1 (Clec-7a)

Dectin-1 is the major CLR involved in the recognition of β-(1,3)-glucan exposed on the surface of germ tubes and hyphae (Hohl et al., 2005; Steele et al., 2005). It is a transmembrane receptor with an extracellular lectin domain and an intracellular tyrosine-based activation (ITAM) motif. Engagement of Dectin-1 with its ligands induces the downstream signalling pathways mediated either by the spleen tyrosine kinase/-caspase recruitment domain

containing protein 9 (SYK/CARD9) or the RAF-1 pathway (Geijtenbeek and Gringhuis, 2009). Both the pathways induce NF-κB activation leading to the production of proinflammatory cytokines.

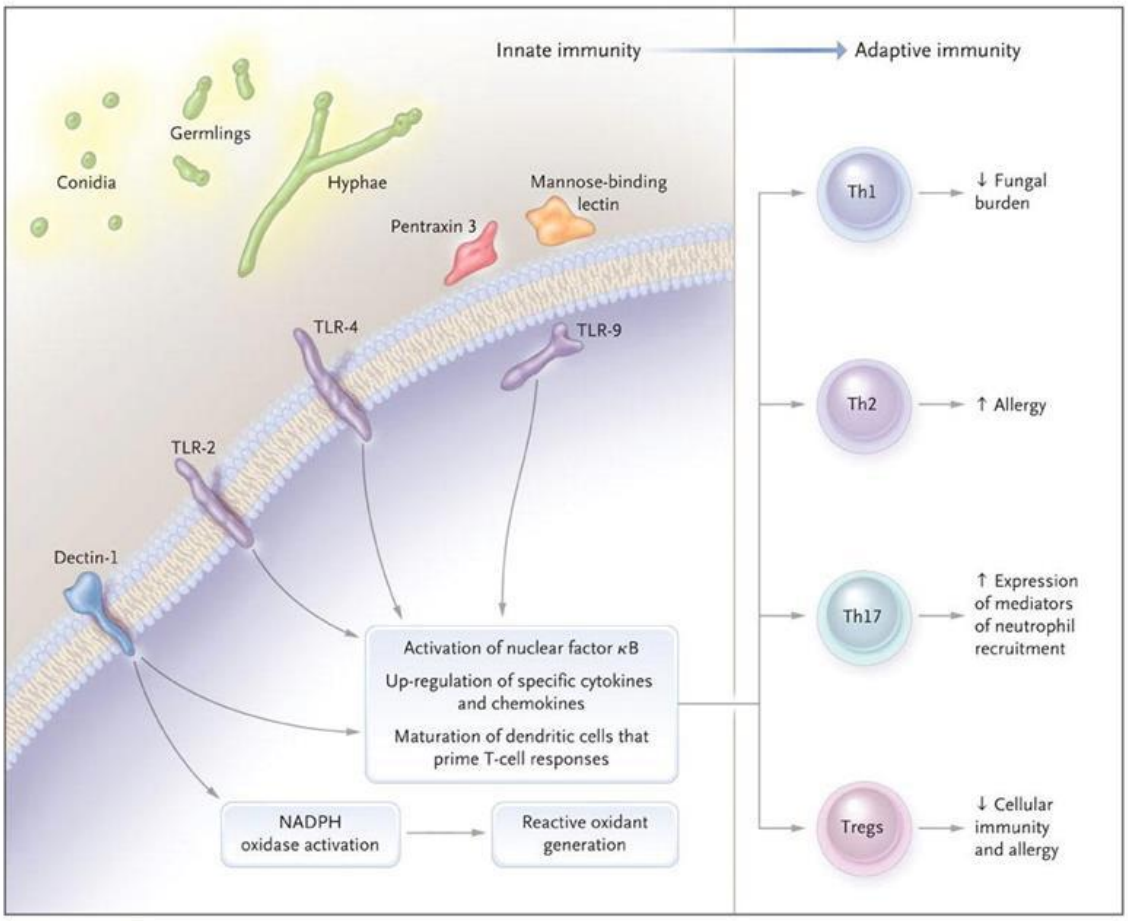

Figure 12. PRRs implicated in sensing and mounting immune response to *A. fumigatus*

Innate immune cells harbour diverse cell membrane PRRs such as dectin-1 and TLRs and soluble PRRs like pentraxin 3 and mannose-binding lectin that sense different morphotypes of *Aspergillus*. Engagement of PRRs with Aspergillus-derived PAMPs leads to the activation of NF-κB and the induction of various cytokines and chemokines. Activation of NADPH oxidase by dectin-1 leads to the generation of reactive oxygen species. DCs activated by PRR-PAMP ligation primes distinct Th subset. (Figure adapted from Segal BH, 2009)

The functional role of Dectin-1 to *A. fumigatus* was initially demonstrated in macrophages, where Dectin-1 blockade abrogated the production of cytokines and chemokines by *A. fumigatus*-stimulated macrophages (Steele et al., 2005). Microarray studies in human DCs have also indicated that Dectin-1 signalling is essential in inducing inflammatory cytokines such as TNF-α and IL-12 (Mezger et al., 2008). Importantly, immunocompetent Clec7a$^{-/-}$ mice are highly susceptible to *A. fumigatus* challenge. Clec7a$^{-/-}$ mice display an enhanced fungal burden in the lungs and are accompanied by decreased inflammatory

mediators. Innate immune cells from Clec7a$^{-/-}$ mice have also an impaired ability to kill *A. fumigatus* conidia (Werner et al., 2009). Further, Dectin-1-mediated signalling plays in an important role in balancing Th1/Th17 response to *A. fumigatus* (Rivera et al., 2011). Of note individuals with mutations in the Caspase Recruitment Domain Family Member (CARD9) gene are susceptible to mucosal and systemic fungal infections (Drummond and Lionakis, 2016; Glocker et al., 2009)

2.6.2 TLR

TLRs are type 1 transmembrane proteins involved in the recognition of PAMPs from diverse classes of pathogens including *A. fumigatus*. TLRs are composed of a LRRs extracellular domain and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain. TLR signalling is mediated by two distinct pathways: MyD88 dependent and MyD88 independent pathways. All TLRs can signal through the MyD88 dependent pathway except for TLR3. MyD88 induces NF-κB activation leading to the production of proinflammatory cytokines. TLR4 can also induce type 1 interferon through the IRF3-dependent pathway (O'Neill et al., 2013).

An important role for TLRs in the recognition of *A. fumigatus* has been suggested. Initial studies indicated that TLR4 and not TLR2 is involved in the recognition of *A. fumigatus* (Wang et al., 2001). However, subsequent studies have demonstrated that both TLR2 and TLR4 are important in sensing *A. fumigatus* (Bellocchio et al., 2004; Braedel et al., 2004; Meier et al., 2003). Interestingly, TLR4 was capable of recognizing either the resting or swollen conidia but not the hyphae. Therefore, the abrogation of TLR4-mediated proinflammatory signals during the germination of conidia to hyphae has been proposed as an evasion mechanism of *A. fumigatus.*(Mambula et al., 2002; Netea et al., 2004, 2003) Importantly, TLR2, TLR4, and the downstream signalling adaptor molecule MyD88 were required for enhancing inflammatory cytokine production and curtailing fungal burden in a murine model of IA. However, TLR2, TLR4, and MyD88 was dispensable in immunocompetent mice (Balloy et al., 2005; Bellocchio et al., 2004; Dubourdeau et al., 2006).

3 Mycobacterium tuberculosis

3.1 General introduction – *M. tuberculosis*

The genus *Mycobacterium* encompasses more than 120 species, which comprises mainly of non-pathogenic species such as *M. smegmatis*, *M. indicus prani*, and *M. fortuitum*. However, the genus also consists of opportunistic and strict pathogens that afflict humans and animals alike. Opportunistic pathogens such as *M. kansasii*, *M. avium*, and *M. haemophilum* are common among immunocompromised individuals. Whereas strict pathogens which include *M. tuberculosis*, *M. leprae*, and *M. ulcerans* are some of the most successful pathogens capable of causing disease in immunocompetent individuals (Cosma et al., 2003). Among all the species of *Mycobaterium*, *M. tuberculosis* is by far the most effective and pernicious human pathogen. According to World Health Organization (WHO) more than 2 billion of the world's population are infected with *M. tuberculosis* with a life-long risk of re-activation. However, more than 90% of the infected population neither develop the disease nor become infectious. Despite the low incidence (~5 - 10%) of individuals developing active disease, deaths caused by *M. tuberculosis* (~1.5 million deaths annually) are among the leading causes of death due to infectious diseases. Although, the global incidence of tuberculosis has dramatically reduced due to the introduction of STOP - TB program by the WHO, the emergence of multi-drug resistant strains (MDR) and extensively drug-resistant strains (XDR) of *M. tuberculosis* and the HIV co-pandemic has aggravated the problem in controlling the tuberculosis epidemic (Kaufmann, 2001; Kaufmann et al., 2014; Pawlowski et al., 2012; Zumla et al., 2014).

3.2 Pathogenesis of *M. tuberculosis*

M. tuberculosis was first discovered by Robert Koch in 1882 as the causative agent of tuberculosis (Koch, n.d.). It is a rod-shaped, non-motile, obligate aerobe, which primarily infects the human lung. *M. tuberculosis* is predominantly transmitted as aerosolized droplets expectorated from an individual with active tuberculosis. The biologically-relevant dose of *M. tuberculosis* infection is unclear; as the infectious dose for an individual is reported to be anywhere between 1 and 200 bacilli and a single aerosol droplet is estimated to contain anywhere between 1 to 400 bacilli (Balasubramanian et al., 1994; Sakamoto, 2012). Human *M. tuberculosis* infection begins when aerosolized droplets containing *Mycobacterium* are inhaled by healthy individuals. The tubercle bacilli enter the alveoli and are taken up by alveolar macrophages, interstitial macrophages, local DCs, and also epithelial cells.

Interestingly, in comparison to other infections there is a delay in the onset of adaptive immune response following *M. tuberculosis* infection (Urdahl et al., 2011). Studies in mice have indicated that *M. tuberculosis* possess a unique ability to establish infection while delaying the adaptive immune response by about 2-3 weeks (Gallegos et al., 2008; Wolf et al., 2008). It has been hypothesized that this "procrastination strategy" employed by *M. tuberculosis* allows the establishment of infection and accumulation of infecting bacilli.

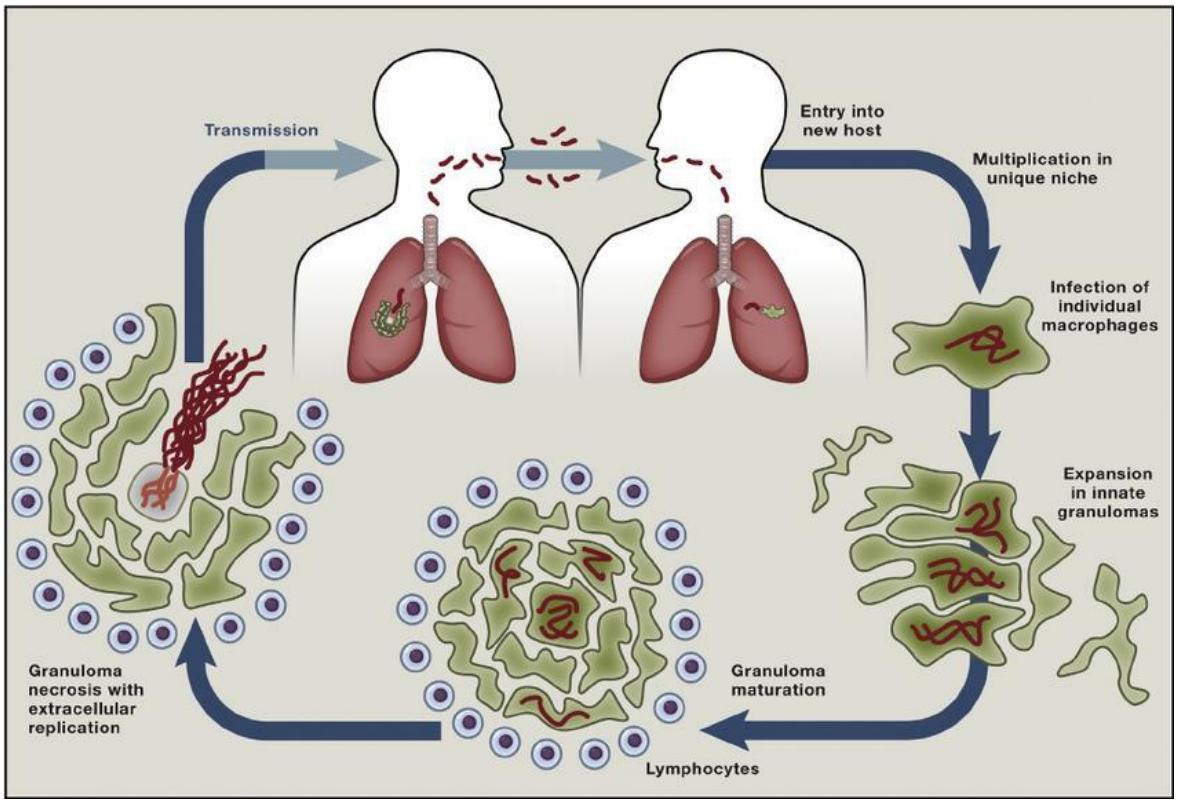

Figure 13. Pathogenic Life Cycle of *M. tuberculosis*

Initiation of *M. tuberculosis* infection begins when aerosols loaded with bacteria expelled from an individual with an active disease gets deposited in the lower lungs of a new host. Subsequently macrophages are recruited to the lung surface, which become infected and transport the bacteria across the lung epithelium to deeper tissues. Further recruitment of macrophages to the original infected macrophage leads to the formation of granuloma, an organized aggregate of differentiated macrophages and other immune cells. The granuloma in its early stages expands infection by allowing bacteria to spread to the newly arriving macrophages. As adaptive immunity develops, the granuloma can restrict bacterial growth. However, under many circumstances, the infected granuloma macrophages can undergo necrosis, forming a necrotic core that supports extracellular bacterial growth and transmission to the next host. (Adapted from Cambier *et al.*, 2014).

Resident phagocytes that interact with *M. tuberculosis* secrete various proinflammatory cytokines and chemokines, which recruit neutrophils and monocytes to the site of infection. The resulting cellular infiltrate form an organized granulomatous parenchymal lesion called the "granuloma", which is the hallmark of pulmonary *M. tuberculosis* infection (Ulrichs and

Kaufmann, 2006). Eventually, adaptive immune cells also infiltrate the granuloma as and when the adaptive immune response gets initiated. The bacteria usually reside in the centre of the granuloma, which is surrounded by concentric layers of macrophages, macrophage-differentiated epithelioid cells, foamy macrophages, DCs, multi-nucleated giant cells, T lymphocytes, B lymphocytes, Neutrophils, fibroblasts, and extracellular matrix components. Dysregulation of infection and inflammation results in the necrosis of the granuloma and later becomes caseous allowing the outgrowth of high numbers of extracellular *M. tuberculosis*. Eventually, liquefaction of the granuloma damages the airway linings, which facilitates the discharge of *M. tuberculosis*. This stage represents the active phase of tuberculosis disease and is highly contagious (Dorhoi et al., 2011). Pathogenesis of *M. tuberculosis* is schematically represented in fig 12.

3.3 Immune response to *M. tuberculosis*

Following *M. tuberculosis* infection, a robust but insufficient innate immune response to contain the infection is generated. The ensuing cellular immunity is absolutely essential in containing the infection. The final outcome of the disease depends on the cumulative strength of all the immune response mounted by the host. Thus, a strong immune response is required to clear or to ensure the confinement of the bacilli within the granulomatous structure. However, the immune response needs to be regulated as excessive inflammation can be detrimental to the host. Different cells of the immune system and immune mediators produced by immune and non-immune cells have multiple redundant roles in a complex network of immune reactions. Concurrently, *M. tuberculosis* is also able to manipulate the host response to form a suitable niche to grow and in parallel promoting immune-mediated damage which is required for transmission of the disease.

3.3.1 Innate immune response to *M. tuberculosis*

Cellular immunity is indispensable in controlling *M. tuberculosis* (Cooper, 2009). However, ample amount of time is required for the initiation of adaptive immune response. During this lag phase, the innate immunity curbs the growth of *M. tuberculosis* by several mechanisms. (Schematic representation of immune response to pathogenic *M. tuberculosis* is depicted in figure 13).

Activation of innate cells by *M. tuberculosis* triggers several effector functions such as secretion of inflammatory cytokines, induction of autophagy, and production of reactive

nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) to kill *M. tuberculosis* (Khan et al., 2016). Although, alveolar macrophages functions as the first line of defense against inhaled *M. tuberculosis*, it also forms a major cellular niche for the replication of bacteria during the initial stages of infection. *M. tuberculosis* is equipped with several antigens, in addition, to the common antigens such as the components of peptidoglycan cell wall and nucleic acid present in bacteria, *M. tuberculosis* also harbour unique antigens, exclusive to

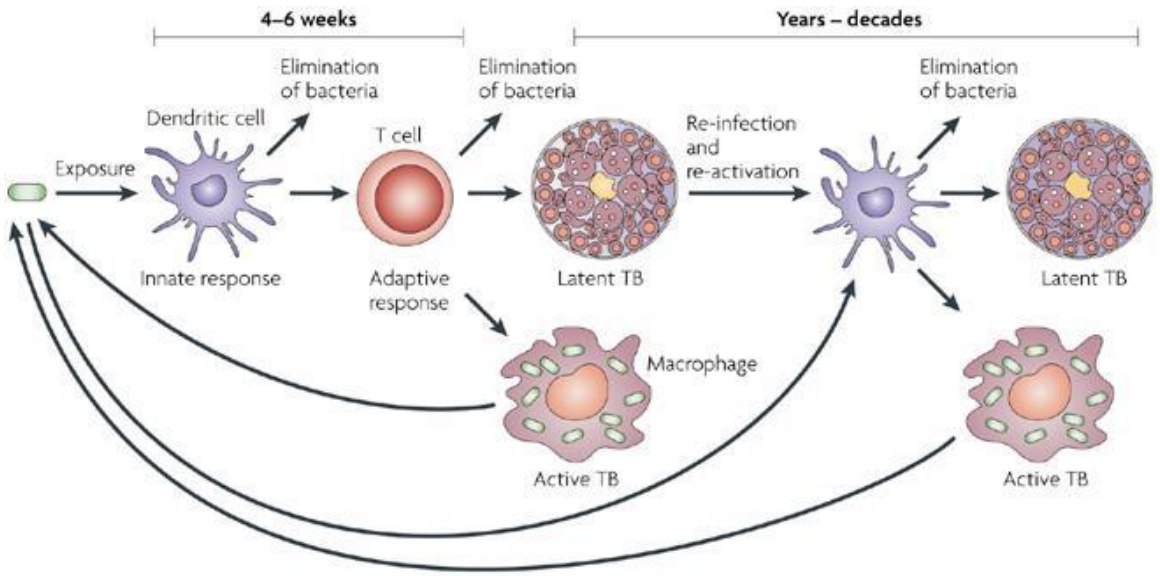

Figure 14. Immunity to *M. tuberculosis*

M. tuberculosis is transmitted by aerosol from individuals with active disease. Bacteria that reach the alveoli of the lung are ingested by macrophages, where they can initiate rounds of intracellular replication and cell lysis. Macrophages are key effector cells in *M. tuberculosis* killing, but incidentally they can also provide a niche for bacterial multiplication. DCs engulf bacteria, or bacterial components, circulate to the draining lymph nodes and prime T cells, which are then recruited to the lungs to orchestrate pathogen control. T cells enhance the antibacterial activity of macrophages by releasing cytokines, such as IFN-γ, which generally results in arrest or clearance of the infection. If T-cell response is insufficient to control the initial infection, clinical symptoms will develop within ~1 year in the form of primary progressive disease. Most individuals develop a T-cell response in the absence of any clinical symptoms, which is defined as a latent infection and carries a risk of secondary disease owing to subsequent reinfection or reactivation of the initial infection. Active diseases is marked by the breakdown of granulomas, which results in uncontrolled replication of *M. tuberculosis* and release of bacteria into the airways and effective aerosol transmission. (Adapted from Young *et al.*, 2008).

mycobacterial species. These antigens are either located on the cell wall such as Lipomannan (LM), lipoarabinomannan (LAM), and its mannosylated form (ManLAM), mycolic acids, phthiocerol dimycocerosate (PDIM); or secreted effector proteins such as early secretory antigenic target-6 (ESAT-6) (Kaur et al., 2009; Majlessi et al., 2015; Mishra et al., 2011). These

antigens are recognized by various PRRs on macrophages and other innate cells. Detection of phosphatidyl-myo-inositol hexamannosides (PIM) and cell wall-associated LAM by TLR2 triggers the activation of macrophages and DCs (Jones et al., 2001; Means et al., 1999; Quesniaux et al., 2004).

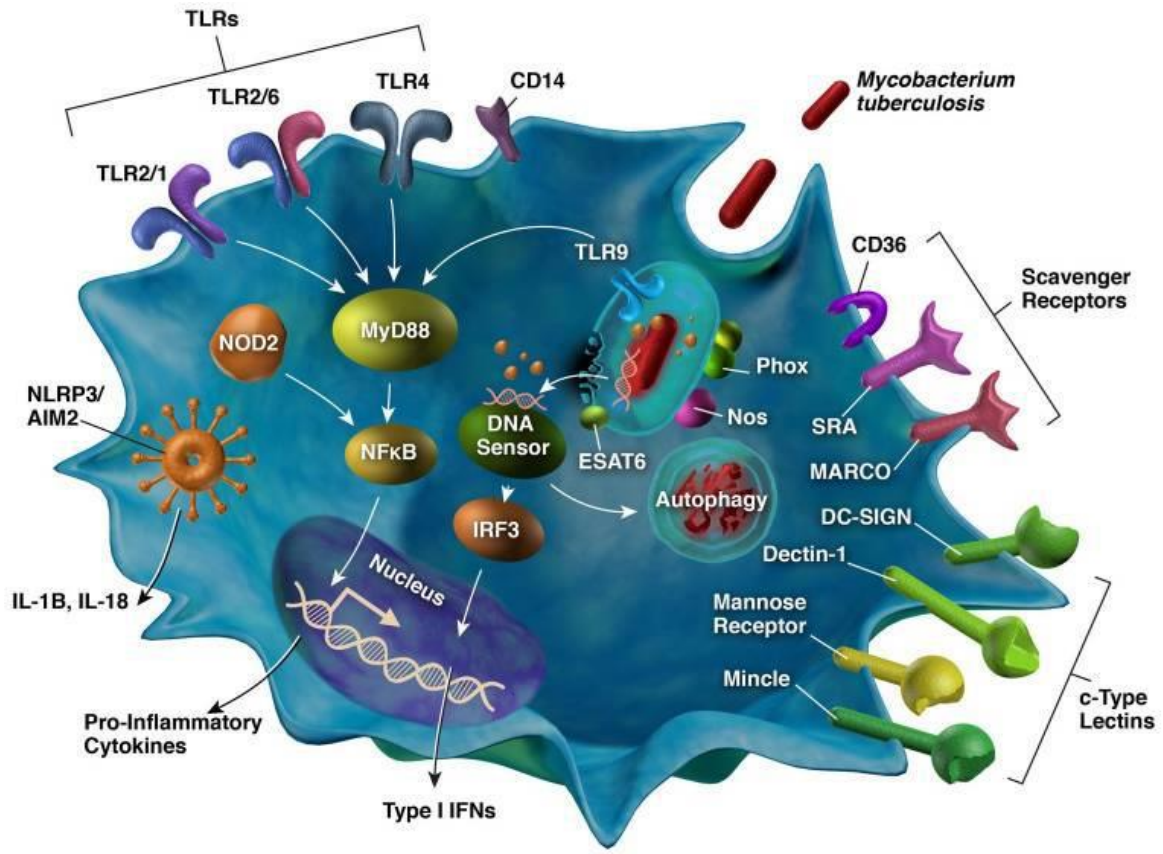

Figure 15. Major PRRs implicated in sensing *M. tuberculosis*.

TLRs, Scavenger receptor (SRs), and CLRs, on the surface of innate cells such as macrophages and DCs are the major PRRs involved in the detection of *M. tuberculosis*. In addition to the membrane associated PRRs, cytoplasmic receptors are involved in the detection of secreted proteins and DNA that access the cytoplasm through phagosomal membrane damage (Adapted from Stamm *et al.*, 2015).

The cell wall proteins such as proline-glutamic acid polymorphic guanine-cytosine-rich sequence (PE_PGRS) family of proteins PE_PGRS 17 (Rv0978c) and PE_PGRS 11 (Rv0754) are sensed by TLR2 on DCs leading to the activation of DCs and enhancing their ability to stimulate CD4+ T cell response (Bansal et al., 2010; Ghorpade et al., 2011; Mukhopadhyay and Balaji, 2011). Mincle, an inducible form of CLR detects trehalose-6,6 dimycolate (TDM), the major cell wall gylco-lipid and induces the production of pro-inflammatory cytokines in macrophages (Ishikawa et al., 2009; Schoenen et al., 2010). Mannose receptor (MR) is involved in the phagocytosis of inhaled *M. tuberculosis* (Schlesinger, 1993). However, MR

signalling upon LAM/ManLAM engagement also leads to the production of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1RA) (Chieppa et al., 2003). The major PRRs implicated in *M. tuberculosis* sensing are depicted in figure 14.

Other anti-mycobacterial effector mechanisms mediated by the innate cells include production of nitric oxide (NO) and RNI. The enzyme inducible nitric oxide synthase (iNOs) catalyses the oxidation of L-arginine to NO and citrulline. NO or its derivatives like peroxynitrite (ONOO-) have critical roles in killing intracellular pathogens. Peroxynitrite is a highly unstable molecule and a potent mediator of oxidative process such as lipid peroxidation. The role of NO is corroborated by the fact that mice lacking iNOs are highly susceptible to *M. tuberculosis* infections (MacMicking et al., 1997). iNOs can be induced upon TLR or NOD engagement and at a later stage by IFN-γ produced by the adaptive cells (Chan et al., 2001).

Autophagy is a major immune mechanism involved in the clearance of intracellular pathogens. It is a catabolic stress response that is involved in targeting the cellular contents for degradation by lysosomes. Replication of intracellular pathogens induces a stress response, which sequesters the pathogens to form a double membrane envelope known as autophagosome. Subsequently autophagosome fuses with lysosomes to form autolysosome to degrade pathogens (Deretic et al., 2009; Gutierrez et al., 2004). Unlike phagosomal degradation where mycobacterium can neutralize the process, degradation via autophagy occurs in the lysosome thus adding an additional barrier. Autophagy is induced by the engagement of mainly endosomal TLRs or NODs such as TLR3, TLR7 and NOD2 (Cooney et al., 2010; Delgado et al., 2008; Delgado and Deretic, 2009). Similar to NO production during later stages of infection, IFN-γ produced by Th1 cells potentiates the induction of autophagy. In addition to the bactericidal mechanism of autophagy; apoptosis and autophagy augment antigen processing and presentation and thereby contributing to the induction of adaptive immunity (Jagannath et al., 2009; Schaible et al., 2003).

3.3.2 Adaptive immune response to *M. tuberculosis*

3.3.2.1 Role of B cells in *M. tuberculosis*

The role of B cells and humoral immunity to intracellular pathogens is generally undermined. However, numerous reports have demonstrated a significant role of B cells and immunoglobulins in shaping the immune response to a number of intracellular pathogens such

as *Salmonella* spp, *Coxiella burnetii*, *Leishmania* spp, *Francisella tularensis* and others (Chan et al., 2014; Culkin et al., 1997; Woelbing et al., 2006). Immunoglobulin (Ig) G has been shown to mediate opsonisation of mycobacterium and enhance phagocytosis by macrophages (Manivannan et al., 2012) (Figure 15). Studies in mice models of BCG infection has shown

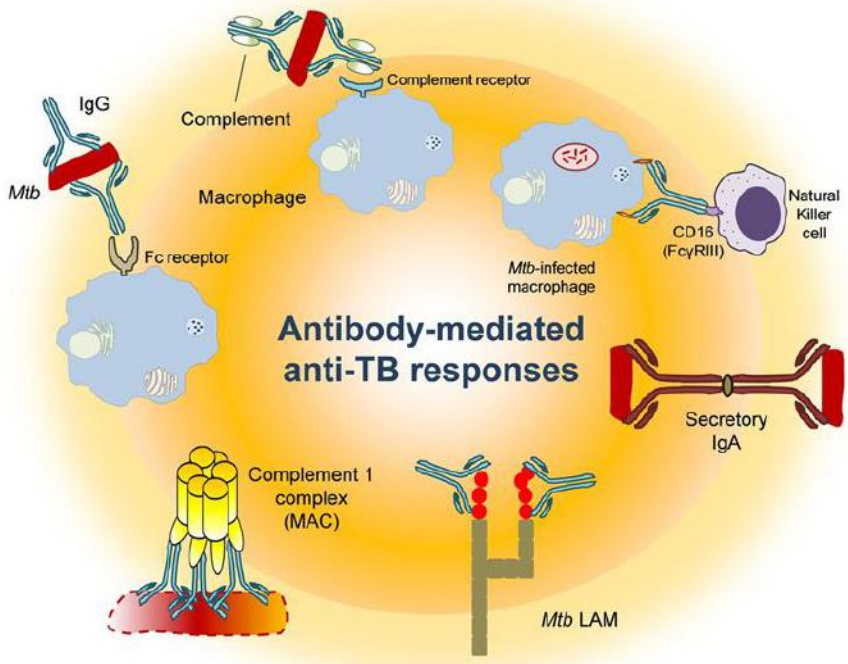

Figure 16. Role of antibodies in anti-*Mycobacterium tuberculosis* infection.

Abbreviations: FcγRIII, Fc gamma receptor III; IgA, immunoglobulin A; IgG, immunoglobulin G; LAM, lipoarabinomannan; MAC, membrane-attack complex; TB, tuberculosis. (Adapted from Rao *et al*., 2015)

that IgA deficient mice are susceptible as they succumbed to pulmonary mycobacterial disease in comparison to their wild type counterparts (Rodríguez et al., 2005). Additionally, passive transfer of monoclonal antibodies against *M. tuberculosis* antigens such as arabinomannan and heparin-binding hemagglutinin as well as the transfer of intravenous immunoglobulin or homologous immune sera, improves the outcome of *M. tuberculosis* infection in mice (Hamasur et al., 2004; Pethe et al., 2001; Roy et al., 2005).

In addition to their role in humoral response, B cells can also function as APC and can produce various inflammatory and immunoregulatory cytokines and chemokines (LeBien and Tedder, 2008). B cells are found at the site of granuloma in both mice and humans and are

believed to mediate antibody-independent B cell functions such as antigen processing and influencing T cell activation (Maglione and Chan, 2009; Tsai et al., 2006). Studies in murine models of *M. tuberculosis* infection and BCG immunization suggested that B cells could regulate Th17 response by modulating the recruitment of neutrophils to lungs (Kozakiewicz et al., 2013). Analysis of B cell functions in tuberculosis patients indicated that, active tuberculosis patients are associated with decreased frequencies of circulating B cells (Joosten et al., 2016). Importantly, *M. tuberculosis* impairs several functions of B cells including proliferation and the ability to secrete cytokines and immunoglobulins (Joosten et al., 2016; Vani et al., 2009). Of note, these impairment of B cell functions could be reversed upon successful treatment (Joosten et al., 2016).

3.3.2.2 Role of T cells in *M. tuberculosis* infection

Adaptive anti-mycobacteria immunity largely depends on T cells. Of the different subsets of T cell, CD4+ T cells have a dominant role in the protection against *M. tuberculosis.* In addition, other T cell subsets, particularly CD8+ T cells, γδ T cells, and CD1-restricted T cell have significant roles in the protection against *M. tuberculosis* (Rojas et al., 1999). Unlike extracellular pathogens, which can be directly targeted by various innate and adaptive immune mechanisms, infection with intracellular pathogens by virtue of residing within the vacuoles of macrophages require the assistance of CD4+ T cells to enhance the clearance of the intracellular pathogens. Antibody-mediated depletion of CD4+ T cells, adoptive transfer of CD4+ T cells, or by disrupting the CD4 gene have confirmed the protective role of CD4+ T cell subset to *M. tuberculosis* infection in murine models (Heuts et al., 2013; O'Garra et al., 2013; Scanga et al., 2000; Tiruviluamala and Reichman, 2002). Furthermore, the modest loss of CD4+ T cells in HIV-infected patients increases the incidence of tuberculosis (Kwan and Ernst, 2011). Indeed, tuberculosis is the leading cause of death in HIV-infected patients. Among different CD4+ T cell subsets, Th1 cells occupy a central role in providing immunity to *M. tuberculosis*. IFN-γ and TNF-α, the major effector cytokines secreted by Th1 cells are the primary immune mediators involved in enhancing bactericidal functions in macrophages. IFN-γ activates several effector functions in macrophages, which includes: stimulation of phagocytosis, phagosome maturation, production of reactive nitrogen intermediates, induction of apoptosis and autophagy, and stimulation of antigen presentation. The role of IFN- γ is corroborated by the fact that individuals harboring mutations in any of the Th1-regulated molecules (IL-12p40 subunit, IL-12Rβ1 chain, IFN-γ, IFN-γ-receptor ligand binding chain,

STAT1) are susceptible to even less pathogenic species of *M. tuberculosis* such as environmental *M. tuberculosis* and Bacillus Calmette-Guerin (BCG) (Casanova and Abel, 2002; Ottenhoff et al., 2002). Thus, the general paradigm for tuberculosis vaccines has largely focused on enhancing the Th1/IFN-γ response (Ottenhoff and Kaufmann, 2012; Skeiky and Sadoff, 2006). However, despite inducing a robust Th1 response, recombinant MVA85A vaccine failed to protect infants from tuberculosis (Tameris et al., 2013). Therefore, it has been suggested that other CD4+ T cell subset such as Th17 may have an important protective role in *M. tuberculosis* infections.

The protective roles of Th17 response in primary *M. tuberculosis* infection is controversial. Initial reports in MDR tuberculosis patients indicated that Th17 response is involved in the exacerbation of the disease rather than mediating protection (Basile et al., 2011). However, more recent reports indicate that *M. tuberculosis* suppresses antigen-specific Th17 response, thus, highlighting a protective role for Th17 response in *M. tuberculosis* infections (Bandaru et al., 2014; Chen et al., 2010; Perreau et al., 2013). Interestingly, individuals with bi-allelic RORC mutations with a defective Th17 response are associated with a compromised Th1 response and are susceptible to fungal and *M. tuberculosis* infections (Okada et al., 2015). Thus, it appears that the protective roles of Th17 response may vary depending on the stage of infection. Th17 response contributes to vaccine-mediated protection and during the earlier stages of infection by recruiting lymphocytes and promoting Th1 response. However, chronic exposure or during the later stages of infection Th17 response may be detrimental due to neutrophil recruitment that can mediate tissue damage leading to immunopathology.

In addition to CD4+ T cells CD8+ T cells, γδ T cells, and CD1-restricted T cells are also implicated in controlling *M. tuberculosis* infection. It is proposed that either cross presentation or *M. tuberculosis* that gets translocated into cytosol are processed by MHC-I and presented to CD8+ T cells (Chen et al., 2004; van der Wel et al., 2007). Classical CD8+ T cells recognize antigens presented in the context of classical MHC-Ia, whereas non-classical CD8 T cells recognize antigens presented in the context of HLA-E (non MHC-Ia) and glycolipids associated with CD1 molecules. CD8+ T cells can secrete IFN-γ, which potentiates macrophage functions and additionally secrete perforin and granzymes which can mediate direct cytotoxic effect on *M. tuberculosis* and can also kill macrophage-infected cells (Lin and Flynn, 2015; Woodworth et al., 2008). *M. tuberculosis* glycolipids and lipoproteins are

presented by CD1 molecules to CD1-restricted T cells (Beckman et al., 1994; Van Rhijn and Moody, 2015). *M. tuberculosis* phosphoantigens are potent stimulators of γδ T cells, which are involved in the early phases of infection (Boom, 1999; Meraviglia et al., 2011; Rojas et al., 2002). γδ T cells can mediate cytotoxic effect and are the major source of IL-17 during the early phase of *M. tuberculosis* infection (Lockhart et al., 2006; Okamoto Yoshida et al., 2010).

3.4 Evasion mechanism of *M. tuberculosis*

Albeit a diverse immune response ranging from innate cells, adaptive cells and immune mediators are elicited during *M. tuberculosis* infection, it is surprising why the immune response at best contains the *M. tuberculosis* in the granuloma and does not eliminate it. Furthermore, it is surprising how *M. tuberculosis* survives in such a hostile immune environment. One explanation has been that the enormous stress response generated by the immune system drives the pathogen into a dormant state. Dormancy is a reversible state where *M. tuberculosis* ceases its replication, but is metabolically active. Interestingly, if immune system wanes, *M. tuberculosis* can sense it and revert back to its infective state. Therefore, in the latent stage of infection, *M. tuberculosis* is said to "sleep" with an "open eye" to detect immune imbalance (Boon and Dick, 2012; Gengenbacher and Kaufmann, 2012). An alternative explanation for *M. tuberculosis* survival is credited to its immune evasion strategies. It is remarkable that a single pathogen is equipped with an armamentarium of mechanism which is capable to restrain, evade and subvert the host protective immune responses. Immune evasion mechanism of *M. tuberculosis* includes inhibition of activation of macrophages and DCs, inhibition of phagosomal maturation, prevention of autophagy and apoptosis, inhibition of antigen presentation, induction of type 1 interferons and enhancement of anti-inflammatory mediators. Additional mechanism of immune evasion includes suppression of protective Th1 responses including IL-12 and IFN-γ and expansion of Tregs (Behar et al., 2010; Flynn and Chan, 2003; Goldberg et al., 2014; Holla et al., 2014).

4 PD-1 – PD-L1 pathway

4.1 General introduction to PD-1 – PD-L1

The programmed cell death (PD-1) protein is a type 1 transmembrane receptor that was first identified in 1992 as a gene upregulated on T-cell hybridomas undergoing cell death (Ishida et al., 1992). It is a member of the Ig superfamily that contains N-terminal IgV-like

domain. It comprises of a transmembrane domain, a cytoplasmic domain with an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (Francisco et al., 2010). A negative regulatory function for PD-1 was first proposed when mice deficient of PD-1 developed spontaneous autoimmune phenotype (Nishimura et al., 2001, 1999). Following this observation several studies have demonstrated a critical role for PD-1 pathway in the maintenance of peripheral tolerance. The ligands for PD-1, programmed death-ligand 1 (PD-L1) and PD-L2 were identified in 2000 and 2001 respectively (Freeman et al., 2000; Latchman et al., 2001). PD-L1 and PD-L2 belong to the B7 family of costimulatory molecules. Members of the B7 family ligands are involved in either delivering a stimulatory or inhibitory signal to T cell (Greenwald et al., 2005). The function of B7 family members is dictated by the receptor on T cells with which they interact. For example, when B7-1 (CD80) and B7-2 (CD86) signal via CD28 on T cell it induces a positive signal. In contrast, signalling of B7-1 and B7-2 via CTLA-4 attenuates T cell response.

Similar too other B7 family members, PD-L1 (B7-H1 or CD274) and PD-L2 (B7-H2 or CD273) contain IgV-like and IgC-like extracellular domains. PD-L1 and PD-L2 have different affinities in binding for PD-1. In fact, PD-L2 has a higher affinity for PD-1 as compared to PD-L1 (Ghiotto et al., 2010; Youngnak et al., 2003). In addition to PD-1, PD-L1 and PD-L2 have additional but different binding molecules; PD-L1 can bind to CD80 whereas PD-L2 has been shown to interact with repulsive guidance molecule b (RGMb) (Butte et al., 2007; Xiao et al., 2014). PD-L1 is broadly expressed on various cells of both hematopoietic and non-hematopoietic origins. Hematopoietic cells that express PD-L1 include T cells, B cells, DCs, macrophages and bone marrow (BM)-mast cells. Further, the expression of PD-L1 can be upregulated upon activation of these cells. Cells of the non-hematopoietic origin that express PD-L1 include vascular endothelial cells, astrocytes, neurons, fibroblastic reticular cells, epithelia, pancreatic islet cells, and cells at sites of immune privilege. In contrast, PD-L2 expression is restricted to fewer cell types than PD-L1. However PD-L2 can be inducibly expressed on DCs, macrophages, B1 cells, memory B cells, and cultured BM-derived mast cells (Francisco et al., 2010). Although, PD-L1 and PD-L2 signalling is involved in attenuating T cell responses, it has also been demonstrated that PD-L1 and PD-L2 can transmit costimulatory signals to T cells. Although, the reasons for the contradictory results are not clear, some studies have suggested that PD-1 – PD-ligands signalling augments T cell proliferation by supressing IFN-γ-induced NO (Tamura et al., 2001; Tseng et al., 2001;

Yamazaki et al., 2005). Alternatively, the possibility of other stimulatory receptor(s) for PD-ligands cannot be ruled out.

4.2 PD-1 – PD-L1 signalling

Activation of PD-1 signalling has a stronger effect on cytokine production (IL-2, IFN-γ, and TNF-α) than on cell proliferation. The maximal PD-1 inhibitory effect is observed on T cells which receive persistent low threshold antigen TCR stimulation. Thus, a strong CD28 and IL-2 signalling can override the signalling events mediated by PD-1-PD-L1 interaction (Carter et al., 2002; Freeman et al., 2000).

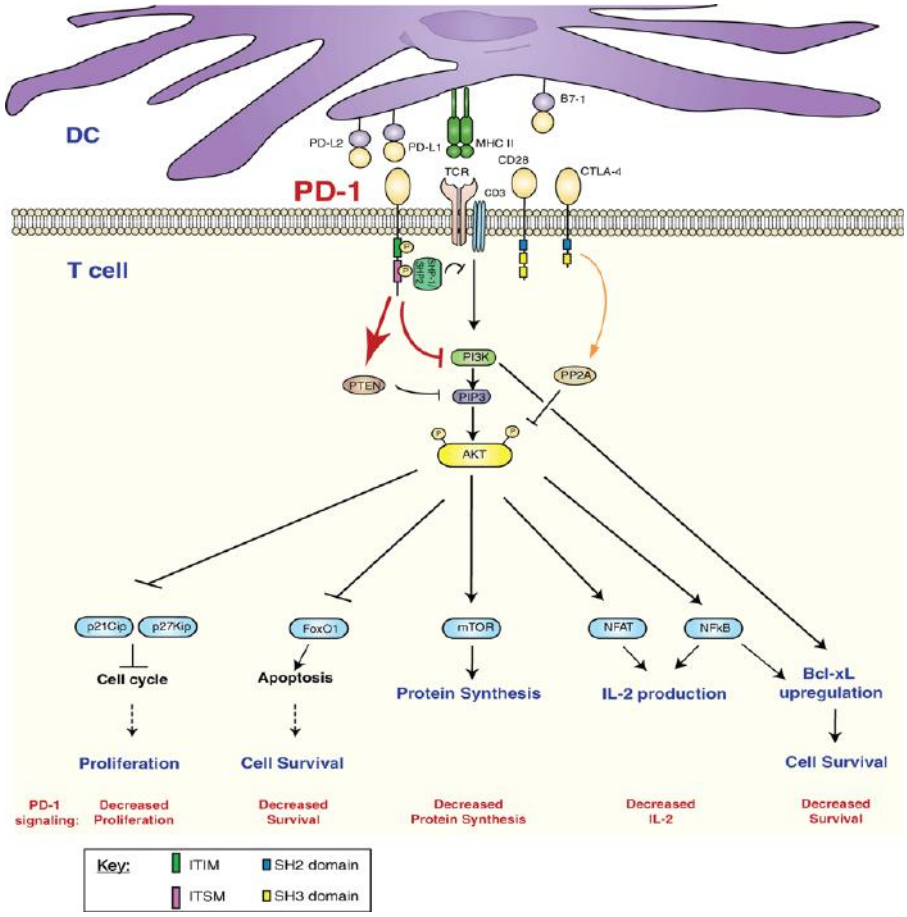

Figure 17. PD-1 signaling.

Engagement of PD-Ls with PD-1 along with TCR signalling leads to tyrosine phosphorylation (P) of the ITIM and ITSM of PD-1. Binding of the ITSM by SHP-1 or SHP-2 results in the dephosphorylation of proximal signaling molecules and augmentation of PTEN expression. This effectively attenuates the activation of the PI3K and Akt pathways. PD-1 signaling may result in decreased T-cell proliferation, survival, protein synthesis, and IL-2 production. (Adapted from Francisco *et al.*, 2010)

PD-L1-PD-1 ligation results in the phosphorylation of the two intracellular tyrosines in the cytoplasmic domain of the PD-1 receptor. Phosphorylation leads to the recruitment of SRC homology 2-domain-containing protein tyrosine phosphatase 1 and 2 (SHP-1 and SHP-2) to the ITSM. SHP-2 dephosphorylates the proximal signalling molecules (CD3ζ and ZAP70) of the TCR signalling thus attenuating TCR signalling. In parallel, ITSM orchestrates the inactivation of AKT by directly inhibiting the induction of phosphatidylinositol-3-kinase (PI3K) activity and by activating PTEN which inhibits PIP3 thus ensuring the inactivation of AKT (Francisco et al., 2010; Parry et al., 2005) (Figure 16). PI3K and AKT are involved in the regulation of glucose transport and glycolysis, thus PD-1 mediated signalling alters the bioenergetics of the cell by altering glucose metabolism. Interestingly, IL-2 can circumvent the PD-1 mediated inactivation of AKT by activating AKT via STAT5 and thereby bypassing the PI3K-dependent AKT activation. Of note, AKT controls diverse cellular functions that govern cell growth (Figure 16). In addition to AKT inhibition, PD-1 signalling also inhibits cell survival gene Bcl-xL, and the transcription factors Tbet and GATA3, which are associated with effector function of Th subsets (Chemnitz et al., 2004; Nurieva et al., 2006). Thus, ligation of PD-1 with PD-L1 inactivates TCR signalling and induces anergy in T cells.

4.3 PD-1 – PD-L1 in tolerance and autoimmunity

A regulatory function for PD-1 in controlling autoimmunity was first proposed when aged mice deficient of PD-1 developed glomerulonephritis (Nishimura et al., 2001, 1999). $Pdcd1^{-/-}$ Balb/c mice displayed enhanced autoantibody against cardiac troponin leading to development of dilated cardiomyopathy. Furthermore, deficiency of PD-1 accelerated autoimmunity in autoimmune-prone backgrounds (Wang et al., 2005). These results support the role of PD-1 pathway in the induction and/or maintenance of tolerance (Figure 17). PD-1/PD-Ls pathway has been implicated in the regulation of both central and peripheral tolerance The PD-1/PD-L1 in particular, plays a critical role at several stages during thymocyte development. During TCRβ rearrangement DN ($CD4^-CD8^-$) thymocytes start to express PD-1 (Nishimura et al., 1996). PD-L1 is broadly expressed on thymic cortex whereas PD-L2 is expressed on the thymic medulla. Thymocytes are also known to expresses PD-L1. The interaction of PD-1 with PD-L1 limits the DP thymocyte ($CD4^+CD8^+$) numbers and the threshold of positive selection during the maturation stage of DN to DP. Loss of PD-1 or PD-L1 enhances the generation of DP thymocytes (Keir et al., 2007). Additionally, the PD-1

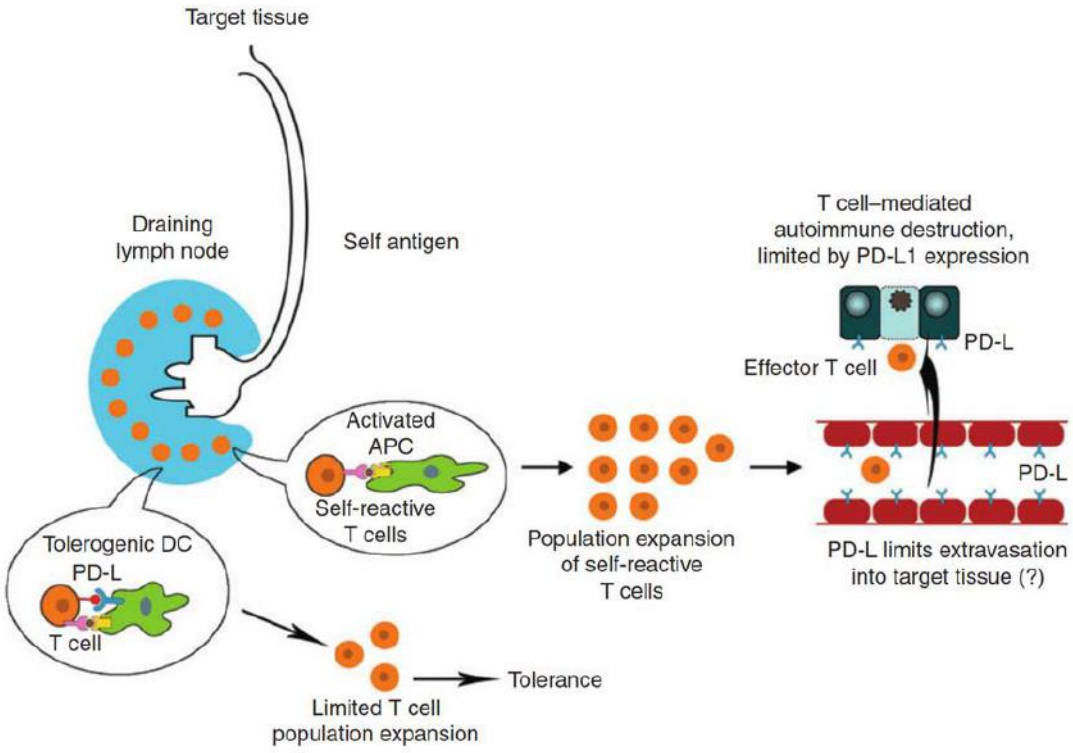

Figure 18. The PD-1–PD-L pathway in controlling autoimmunity.

The PD-1–PD-L1 pathway regulates both the induction and the maintenance of peripheral tolerance. PD-Ls on tolerogenic DCs can induce T cell tolerance. After initial T cell activation, PD-1–PD-L1 interactions can limit self-reactive T cell proliferation and cytokine production. Effector functions of self-reactive T cells that migrate to the target may be limited by PD-L1 expressed on nonhematopoietic tissue cells. PD-L1 seems to have a unique function in maintaining tolerance in the target tissue. (Adapted from Sharpe *et al*., 2007)

signalling contributes to the negative selection process, however this process is not clearly understood (Blank et al., 2003). Overall, the PD-1/PD-L1 signalling contributes to the central tolerance by regulating both the positive and negative selection of thymocytes.

PD-1-PD-L1 interactions have been shown to play an essential role in the initial phases of activation and expansion of self-reactive T cells. The generation of self-reactive T cells is regulated by the initial interaction of T cells with APCs, such as DCs, by displaying self-antigen on resting DCs. PD-1 has been shown to play an important role in inhibiting the responses of self-reactive T cells and curtailing its interaction with DCs (Probst et al., 2005). Loss of PD-1 on antigen-specific T cells increases the CD8+ T cell responses to antigen-bearing resting DCs. Additionally, PD-1/PD-L1 interaction also influences self-reactive T cell effector functions upon antigen encounter. For example, loss or blockade of either PD-1 or PD-L1 in a mouse

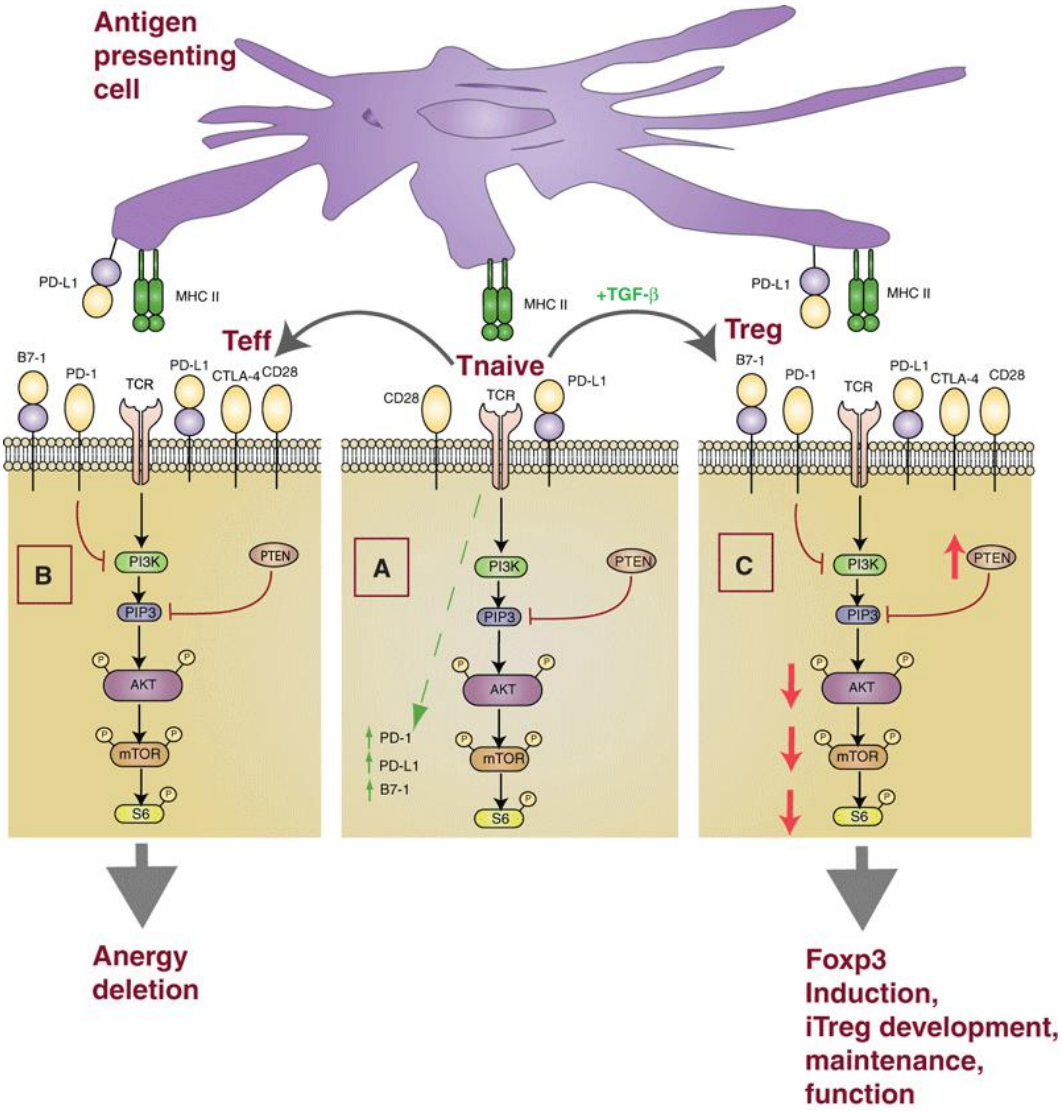

Figure 19. Fates of naive T cells following PD-1 ligation

(A) Upon TCR engagement of a naive CD4+ T cell, PD-1 and PD-L1 are upregulated. (B) The PD-L1 pathway inhibits the downstream PI3K/AKT pathway in naive T cells resulting in functional inactivation of naive T cells and inhibition of effector T-cell differentiation and function. (C) In the presence of TGF-β, the PD-1 pathway attenuates the PI3K/AKT pathway, preferentially biasing naive T-cell programming towards iTreg development and survival. (Adapted from Francisco *et al*., 2010)

model of diabetes exacerbates diabetes with accelerated insulitis (Ansari et al., 2003; Keir et al., 2006). Of note, blockade of either PD-1 or PD-L1 and not CTLA-4 or PD-L2 rescued anergy in islet-antigen-specific T cells, suggesting a unique function of the PD-1/PD-L1 pathway in maintaining anergy (Fife et al., 2006). Figure 18 depicts the fate of naïve T cell upon PD-1-PD-L1 ligation.

PD-L1 Ig in the presence of TGF-β induces the generation of iTregs from naive CD4+ T cells. Moreover PD-L1 Ig enhances the suppressive capacities of iTregs (Francisco et al., 2009). In line with this, PD-L1 deficient APCs or blocking PD-L1 compromises the ability of APCs in inducing Tregs, thus highlighting the role of PD-L1 in the development of Tregs (Francisco et al., 2009). However, it is not clear how the PD-L1 pathway augments suppressive functions in iTregs, but the mechanism of iTreg generation was attributed to the inhibition of Akt/mTOR pathway with a concomitant induction in PTEN. Further, murine studies have suggested an important role for PD-L1 on Tregs in mediating foeto-maternal tolerance. Lack of PD-L1 resulted in decreased allogeneic foetal survival rates with enhanced Th1 response to alloantigens (Guleria et al., 2005). Additionally, foetal survival was improved with adoptive transfer of Tregs from wild type mice but not PD-L1-deficient mice (Habicht et al., 2007). These results thus suggested that PD-L1 on Tregs was necessary in controlling materno-foetal tolerance. As both APCs and Tregs express PD-L1, the inhibitory effect of APCs on effector T cells mediated by PD-1/PD-L1 pathway may also occur between Tregs and effector T cells. One line of evidence comes from a study, which demonstrated that PD-L1 engagement converted effector Th1 cells into iTregs (Amarnath et al., 2011).

4.4 Role of PD-1 – PD-L1 signalling during infections

As the PD-1/PD-L1 pathway is majorly involved in restraining effector immunity, several pathogens have been implicated in exploiting the PD-1/PD-L1 pathway to evade host protective immune response. The role of PD-1 signalling in infection was demonstrated in a murine model of chronic lymphocytic choriomeningitis virus (LCMV) infection. Genome wide microarray of exhausted vs functional virus-specific CD8+ T-cells, revealed a significant induction of PD-1 expression on exhausted virus-specific CD8+ T cells, in comparison with functional LCMV-specific CD8+ T cells (Barber et al., 2006). In vitro blockade of PD-1 in these exhausted CD8+ T cells restored effector functions of CD8+ T cells, with enhanced proliferation, cytotoxicity, cytokine production and a decrease in viremia (Barber et al., 2006) (Figure 19). Moreover, as and when the infection is cleared the expression of PD-1 decreased, which also augmented the generation of functional memory T cell population.

Subsequent studies have extended the initial observations in mice to humans; the extent of PD-1 expression is higher on virus-specific (HIV, HBV, and HCV) T cells (Boettler et al., 2006; Day et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). In HIV infection, the PD-1/PD-L1 pathway was shown to contribute to T cell dysfunction with enhanced viral titres

(Trautmann et al., 2006).Similar to LCMV infection blockade of PD-1/PD-L1 increased the proliferation and survival capacity of HIV-specific CD4+ and CD8+ T cells with enhanced production of cytokines and cytotoxic molecules in response to cognate antigen (Day et al., 2006; Petrovas et al., 2006). Notably, the expression of PD-1 on virus-specific CD8+ T cells decreased in patients who have resolved HCV infection but the expression of PD-1 remained high in patients who progressed to chronic HCV infection (Urbani et al., 2006). Similarly, the expression of PD-1 decreased on HBV-specific CD8+ T cells in patients who controled infection (Boettler et al., 2006).

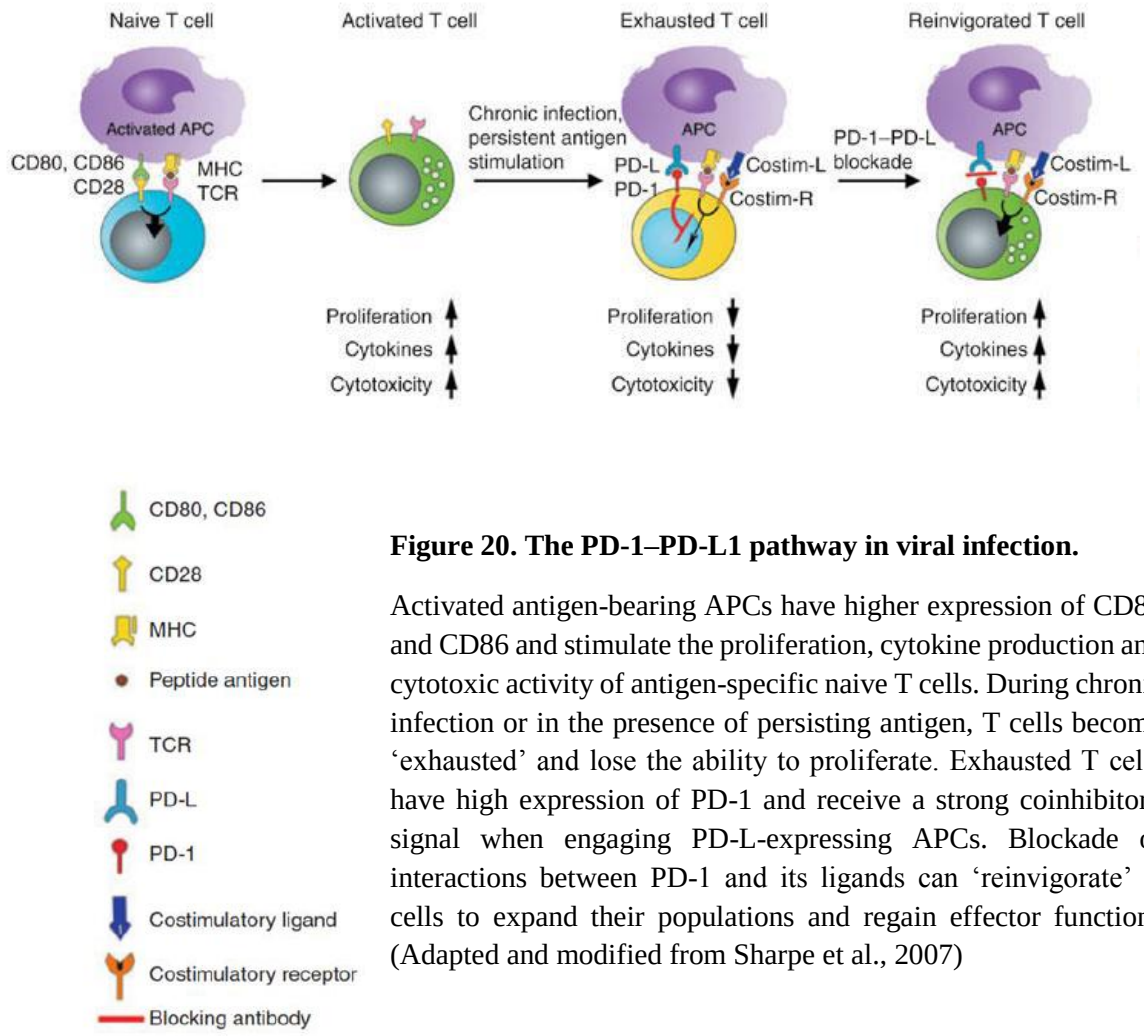

Figure 20. The PD-1–PD-L1 pathway in viral infection.

Activated antigen-bearing APCs have higher expression of CD80 and CD86 and stimulate the proliferation, cytokine production and cytotoxic activity of antigen-specific naive T cells. During chronic infection or in the presence of persisting antigen, T cells become ‘exhausted’ and lose the ability to proliferate. Exhausted T cells have high expression of PD-1 and receive a strong coinhibitory signal when engaging PD-L-expressing APCs. Blockade of interactions between PD-1 and its ligands can ‘reinvigorate’ T cells to expand their populations and regain effector functions (Adapted and modified from Sharpe et al., 2007)

The effector function of T cell depends on the expression of PD-1, which oscillates with respect to the inflammatory stimuli in a way consistent with the involvement of PD-1 in limiting tissue damage. Therefore, although PD-1/PD-L1 signalling dampens effector immune response during infections, PD-1 signalling is pivotal in monitoring immunopathology. Studies

in animal models of infection suggest that PD-1$^{-/-}$ mice clear adenovirus infection more efficiently, but also develop more severe hepatocellular injury than the wild type mice (Iwai et al., 2003). Also, perturbing the PD-1/PD-L1 pathway by administration of PD-L1 specific-blocking antibody in mouse model of herpes stromal keratitis resulted in an exacerbated keratitis, but augmented HSV-1-specific effector CD4+ T cell functions (Jun et al., 2005). Furthermore, in vitro blockade of PD-1 in LCMV-specific exhausted CD8+ T cells although restored effector T cell function, PD-L1 mice were highly susceptible to LCMV infection (Barber et al., 2006). In contrast, their wild-type counterparts survived and developed chronic infection. These studies thus highlight the importance of PD-1/PD-L1 signalling in limiting immunopathology during infections.

Bacterial pathogens and parasitic worms also exploit PD-1 pathway to evade host immunity. PD-1/PD-L interactions mediate suppressive effects of macrophages during *Schistosoma mansoni* and *Taenia crassiceps* infections (Smith et al., 2004; Terrazas et al., 2005). PD-L1 and PD-L2 have distinct functions in regulating the immune response to *Leishmania*. PD-L1$^{-/-}$ mice have lower lesion development and reduced parasite burdens, whereas PD-L2$^{-/-}$ mice develop exacerbated disease with enhanced parasite burdens (Liang et al., 2006). Interestingly, PD-L1 mice had lower Th2 response, which was attributed to the resistance to *L. Mexicana* infection. Therefore, PD-L1 was suggested to modulate Th2 response, however, the mechanism of Th2 regulation by PD-L1 is unclear (Liang et al., 2006). Chronic *Helicobacter pylori* infection leads to gastroduodenal ulcers and gastric cancer. *H. pylori* infection induces PD-L1 expression on gastric epithelial cells, which play an important role as APCs in instructing T cells response (Das et al., 2006). PD-L1 is also upregulated in gastric biopsies from *H. pylori*-infected individuals in comparison with samples from uninfected people (Das et al., 2006). Intracellular pathogens such as *Salmonella* and *Mycobacterium* also benefit from exploiting the PD-1 axis. It has been demonstrated that *Salmonella* uses B cells as Trojan horse to cause infection. Recent studies indicate that one of the mechanisms for the survival of *Salmonella* within B cells is by inducing PD-L1 on B cells, which subsequently blunts CD8+ T cell responses (Lopez-Medina et al., 2014). *M. tuberculosis*-induced PD-L1 has been implicated in the expansion of Tregs and suppression of Th1 responses (Alvarez et al., 2010; Periasamy et al., 2011; Trinath et al., 2012). Although, PD-1/PD-L1 signalling has been implicated as an immune evasion strategy employed by *M. tuberculosis*, complete abrogation of the PD-1 signalling exacerbates infection (Lázár-Molnár et al., 2010).

4.5 Role of PD-1 – PD-L1 signalling in cancer

The first evidence for the PD-1 – PD-L1 pathway in dampening immunosurveillance to tumors came from the observation that overexpression of PD-L1 on mouse plasmacytoma cell line inhibited the cytolytic activity of CD8+ T cells by engaging with its receptor PD-1 (Iwai et al., 2002). Further, PD-L1+ tumor cells eliminated effector T cells by inducing apoptosis. Importantly, PD-L1+ myeloma cells failed to produce tumors in PD-1-deficient mice and perturbing the PD-1 axis activated CD8+ T cells, which could kill tumors. (Iwai et al., 2002). Subsequent studies from several groups have demonstrated the role of role of PD-1/PD-L1 pathway in different tumor models such as ovarian cancer, squamous cancer, and others in suppressing anti-tumor immune response (Baumeister et al., 2016; Curiel et al., 2003; Strome et al., 2003). Tumour-associated APCs control their anti-tumour T-cell response by PD-1/PD-L1 pathway. It has been shown that PD-L1 blockade by mAb enhances DC-mediated T-cell activation (Brown et al., 2003). However, most tumor cell lines from both humans and mouse expresses very low levels of PD-L1 despite the fact that freshly isolated tumor cells express high levels of PD-L1.

In recent years it has become evident that tumor cells evade anti-tumor immune response by altering their phenotype and rewire the signalling pathways. In this context, the PD-1-PD-L1 signalling-mediated tumor evasion has been described as an “adaptive immune resistance” mechanism (Chen and Han, 2015). This rational stems from the observation that under haemostasis normal tissue seldom express PD-L1, but inflammatory cues such as IFN-γ can significantly induce PD-L1 on most nucleated cells. Therefore, IFN-γ production by tumor-infiltrating lymphocytes (TILs) although plays an important role in tumor elimination, it also contributes to tumor evasion by inducing PD-L1 on cancer cells. This hypothesis is supported by the observation that PD-L1 expression is detected only in cells that are adjacent to T cells in the tumor environment (Taube et al., 2012). Further, a strong correlation between PD-L1 expression and TILs was observed in human melanocytic lesions. Notably, IFN-γ was detected at the interface of TILs and PD-L1+ cells whereas it was undetectable within PD-L1– tumors (Taube et al., 2012).

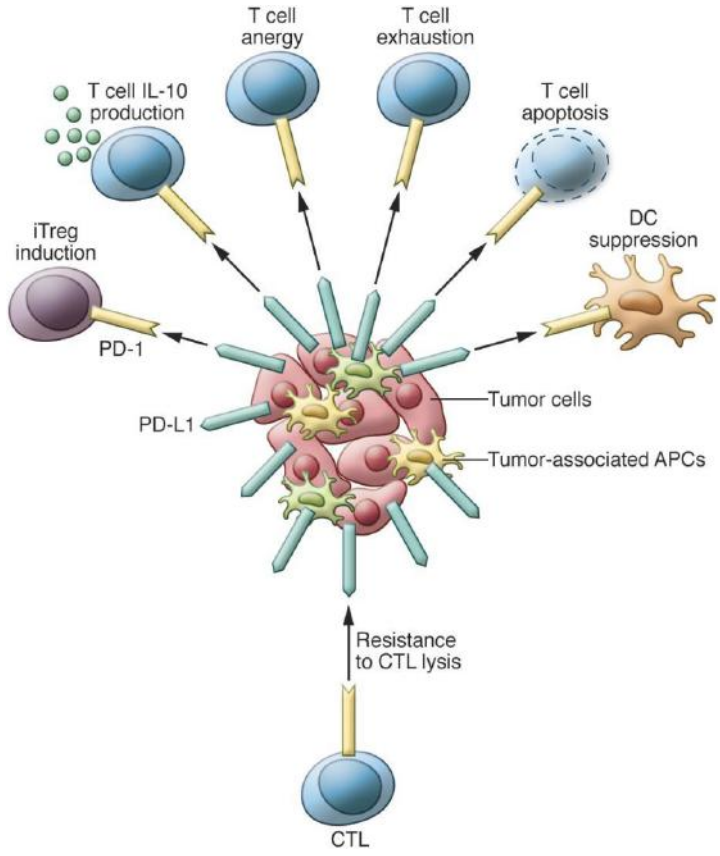

Figure 21. PD-1 – PD-L1 in tumor evasion.

Tumor cells and other cells in tumor microenvironment can express high levels of PD-L1, which results in suppressed immunity upon interaction with PD-1. PD-L1–expressing cells use multiple mechanisms to suppress tumor immunity. PD-L1 tumor cells and antigen-presenting cells can induce T cell apoptosis, anergy, functional exhaustion, and IL-10 production. They can also mediate DC suppression and induce differentiation of Tregs. PD-L1 can also act as a molecular shield on tumor cells and protect tumor cells from lysis by CTLs. (Adapted from Chen and Han, 2015)

More recently it was demonstrated that CD8+ T cells in the melanoma tumor microenvironment induce the expression of PD-L1, Tregs and IDO, which potentiate immune suppression in the tumor microenvironment (Spranger et al., 2013). These studies thus underscore the role of PD-1/PD-L1 pathway in conferring adaptive immune resistance to tumors. Furthermore, these studies provide an explanation for the ineffectiveness of certain immunotherapies such as cancer vaccines, which are primary aimed at enhancing IFN-γ responses. Figure 20 depicts the various mechanisms employed by cancer cell to evade host immune response.

Objectives of my Thesis

The identification that PD-1 has an essential role in maintaining peripheral tolerance stemmed an interest in deciphering the role of PD-1 axis in different pathologies such as autoimmunity, cancer, and infection. Ligation of PD-L1 with PD-1 attenuates T cells responses by inducing anergy, supressing effector T cell responses, and by inducing Tregs. While these mechanisms have a crucial role in repressing autoimmune responses; cancer cells and pathogens can benefit from the PD-1 axis by curbing protective host immune response. Several groups have demonstrated that the PD-1/PD-L1 mediated suppressive mechanisms is a major tumor evasion strategy employed by cancer cells, and blockade of PD-1/PD-L1 restores anti-tumor immunity. In contrast, the PD-1 pathway although, suppresses the effector immune responses targeted at the pathogen, it also plays an indispensable role in curtailing immunopathology. Thus, the PD-1/PD-L1 pathway plays a critical role in regulating the delicate balance between immunopathology and protective immunity during infections.

Pulmonary infections caused by respiratory pathogens are among the major causes of mortality and morbidity worldwide. Annually ~3.5 million deaths are attributed to lower respiratory tract infections. Although, significant advances have been made in intervening and treating most of these diseases, pathogens have evolved various immune evasion strategies to supress and subvert host immune responses. Thus, it is pertinent to decipher both the immune response elicited by the host and the evasion strategy employed by pathogens for developing effective therapies in combating respiratory infections. In view of latest developments on the role of PD-1/PD-L1 axis in suppressing effector T cell responses and success of immune check point targets in clinics, I investigated the role of PD-L1 pathway in modulating immune response to *M. tuberculosis* a bacterial pathogen and *A. fumigatus* an opportunistic fungal pathogen. Specifically, my thesis was aimed at

- Deciphering the role of PD-L1 in regulating immune response to cell wall polysaccharide of *A. fumigatus.*
- Delineating the role of PD-L1 on dendritic cells in modulating Th1 response to *M. tuberculosis.*
- Dissecting the role of PD-L1 on innate cells in modulating Th17 response to *M. tuberculosis* and *M. tuberculosis*-derived antigen fractions.
- Delineating the role PD-L1 and cyclooxygenase-2 in mediating Treg responses to *M. tuberculosis.*

Results

Aspergillus fumigatus cell wall α-(1,3)-glucan stimulates regulatory T cell responses by inducing PD-L1 expression on human dendritic cells

(Article I)

Stephen-Victor E, Hegde P, Fontaine T, Beauvais A , Holla S,Balaji KN, Kaveri SV, Latgé JP, Aimanianda V, Bayry J.

Aspergillus fumigatus cell wall α-(1,3)-glucan stimulates regulatory T cell responses by inducing PD-L1 expression on human dendritic cells

Running Title: PD-L1-dependent induction of Tregs by α-(1,3)-glucan

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Abstract

α-(1,3)-Glucan is an amorphous component of the *Aspergillus fumigatus* cell wall. Its major proportion among cell wall polysaccharides and its surface localization during growth suggest that α-(1,3)-glucan has a critical role in modulating the host innate immune cells and T cell responses. However, human innate cell response to α-(1,3)-glucan, and ensuing $CD4^+$ T cell polarization are poorly characterized. In this report we show that α-(1,3)-glucan induces maturation of human dendritic cells (DCs) and secretion of various immunoregulatory cytokines. α-(1,3)-Glucan-induced activation of DCs was dependent in part on TLR2. The minimum α-(1,3)-oligosaccharides chain-length required to stimulate DCs was in the range of 10-14 monomeric units (dp). However, the short α-(1,3)-oligosaccharides (dp4-8 and 6-10) efficiently blocked DC-stimulatory ability of α-(1,3)-glucan. Analysis of polarization of $CD4^+$ T cells revealed that α-(1,3)-glucan-stimulated DCs induce programmed death-ligand 1 (PD-L1)-dependent $CD4^+CD25^+FoxP3^+$ regulatory T cell (Treg) responses. Notably, blockade of PD-L1 on DCs led to enhanced secretion of IFN-γ suggesting that PD-L1 acts as negative regulator of α-(1,3)-glucan-mediated protective immune responses. Taken together, these results demonstrate that *A. fumigatus* cell wall polysaccharides including α-(1,3)-glucan diversify the immune response by their distinct abilities to polarize-specific $CD4^+$ T cell responses. Our results also provide a rational for the exploitation of immunotherapeutic approaches that target PD-1–PD-L1 axis to restore protective T cell response to *A. fumigatus*.

Introduction

Aspergillus fumigatus is the most ubiquitous aerial human fungal pathogen. Owing to their small size (2-3 μm), the airborne spores (conidia) of *A. fumigatus* enter the human lung-alveoli through breathing air. In immunocompetent individuals, these conidia are eliminated by resident phagocytes whereas in the immunocompromised host, conidia germinate and invade the host resulting in a fatal condition, invasive aspergillosis. *A. fumigatus* also causes a number of other diseases such as lung/sinus aspergilloma and allergic broncho-pulmonary aspergillosis in the immunocompetent individuals (1, 2).

In addition to macrophages and neutrophils, immunity towards *A. fumigatus* infection requires co-ordinated action of dendritic cells (DCs) and adaptive immune components. The central role of DCs in controlling microbial immunity is linked to the ability of these cells to modify their properties in response to external stimuli. DC maturation involves multiple alterations, including changes in antigen processing, presentation and expression of major histocompatibility complex molecules, co-stimulatory molecules, cytokines and chemokines that affect their ability to attract and regulate the differentiation and activation of T cells. It has been demonstrated that DCs could internalize both *A. fumigatus* conidia and hyphae, and undergo functional maturation to instruct local and peripheral $CD4^+$ T cell reactivity to the fungi (3-5). Different families of pattern recognition receptors (PRRs) such as TLR, C-type lectin receptors (such as DC-SIGN and Dectin-1) and pentraxins on these cells participate in the recognition of germinating *A. fumigatus* (5-15).

Aspergillus cell wall is a complex and dynamic structure, and major source of pathogen-associated molecular patterns (PAMPs). The composition and localization of the cell wall components evolve constantly in response to environmental stimuli and vary with the morphotypes of *A. fumigatus*. Polysaccharides constitute nearly 90% of the cell wall and mainly composed of branched β-(1,3)-glucan, α-(1,3)-glucan, chitin, galactomannan and morphotype-specific galactosaminogalactan (GAG) (16). Several recent reports have demonstrated that β-(1,3)-glucan is the major immunogenic motifs of germinated *A. fumigatus* conidia and hyphae that is recognized by Dectin-1 on innate cells, inducing inflammatory responses as well as Th17/Th1 responses (5, 17-19). Chitin, galactomannan and galactosaminogalactan also exert immuno-regulatory functions (14, 20-24). However, based on the *in vitro* chemical analysis of germinating conidia and mycelia, it was proposed that β-(1,3)-glucan and chitin are not immediately accessible to immune system. On the contrary,

outer cell wall layer that compose mainly of alkali-soluble polysaccharides including α-(1,3)-glucan, galactomannan, galactosaminogalactan and *N*-glycosylated proteins mask β-(1,3)-glucan and chitin (16). Immuno-cytochemical analysis of the cell wall of swollen conidia demonstrated the presence of α-(1,3)-glucan at the cell surface, indicating that α-(1-3)-glucans are surface exposed during germination process (25).

α-(1,3)-Glucan is an amorphous component of the *A. fumigatus* cell wall and acts as a cement for other constituents of the cell wall (26). In addition, outer α-(1,3)-glucan is associated to the infective phase of several pathogenic yeasts including *Cryptococcus neoformans* and *Histoplasma capsulatum* (27, 28). Thus, the surface localization of α-(1,3)-glucan and its major proportion (about 40% in the mycelia and 19% in the conidia) among cell wall polysaccharides (29) suggest that α-(1,3)-glucan has a critical role in modulating the innate cells and T cell responses. However, the human innate immune cell response towards α-(1,3)-glucans, and ensuing $CD4^+$ T cell polarization are poorly characterized.

In this study we show that α-(1,3)-glucan induces activation of human DCs with a capacity to induce $CD4^+FoxP3^+$ regulatory T cell (Treg) responses. Blocking experiments suggest that α-(1,3)-glucan-induced programmed death-ligand 1 (PD-L1) on DCs mediate Treg polarization. α-(1,3)-Glucan could promote Th1 (IFN-γ) responses only when PD-L1 was blocked suggesting that PD-L1 acts as a negative regulator of α-(1,3)-glucan-mediated protective immune responses. We identified minimal degree of polymerization required for the α-(1,3)-glucan to induce maturation of DCs. Our results also demonstrate that α-(1,3)-glucans purified from either mycelia or conidia have similar ability to induce maturation of DCs and that short α-(1,3)-oligosaccharides inhibit α-(1,3)-glucan-mediated DC-maturation.

Materials and Methods

Reagents and antibodies

PE-conjugated MAbs to CD80 (clone L307.4), CD83 (clone HB15e), CD273(clone MIH18), CD275 (clone 2D3/B7-H2), IL-4 (clone MP4-25D2); FITC-conjugated MAbs to CD86 (clone 2331 (FUN-1)), CD274 (Clone MIH1), CD25 (clone M-A251), IFN-γ (clone 4S.B3); APC-conjugated MAbs to CD83 (clone HB15e), HLA-DR (Clone G46-6); Alexa 700-conjugated MAbs to CD4 (clone RPA-T4) were from BD Biosciences. PE-conjugated MAb to CD40 (clone MAB89) and CD252 (clone 11C3.1) were from Beckman Coulter and Biolegend, respectively. PE-conjugated MAbs to IL-17A (clone eBio64CAP17), APC-

conjugated MAbs to FoxP3 (clone 236A/E7) and Fixable Vibility Dye eFluor 506 were from eBioscience.

Blocking MAbs to human TLR-2 (clone 383936), DC-SIGN (clone 120507), Dectin-1 (clone 259931) were from R&D Systems; anti-TLR4 (clone HT52), anti-PD-L1 (clone MIH1) and isotype control antibodies were from eBioscience. Mannan was obtained from Sigma–Aldrich.

A. fumigatus strains

The clinical isolate CBS144.89 was the wild-type (WT) *A. fumigatus* strain used (30) and was maintained on 2% malt-agar slants at ambient temperature.

Extraction and purification of α-(1,3)-glucan from the A. fumigatus morphotypes

Conidia were harvested from the 10-15 days cultured malt slants, washed twice with 0.05% aqueous Tween and used for the α-(1,3)-glucan extraction-purification. For mycelial culture, conidia were inoculated into Sabouraud liquid medium, incubated at 37°C in a shaken incubator for 24 h; mycelia were collected upon filtration and washed thoroughly with water. Briefly, conidia and mycelia were disrupted with glass beads (0.5-mm and 1 mm diameter for conidia and mycelia, respectively) in a FastPrep (MP Biomedicals); the cell wall fractions were separated upon centrifugation (4000 rpm, 10 min) and washed twice with water. Further, the cell wall fractions were boiled with TRIS (50 mM)-EDTA (50 mM)-SDS (10%)-β-mercaptoethanol (40 mM) reagent, twice and each time supernatant was discarded after centrifugation. The resulting cell wall fractions, containing only polysaccharides, were washed thoroughly with water. Following, these fractions subjected to alkali-fractionation upon incubating with NaOH (1 M)-$NaBH_4$ (0.5 M) reagent at 65°C for one hour, twice and each time the supernatants after centrifugation (alkali-soluble (AS)-fraction) were collected, neutralized with 2% acetic acid, dialyzed against water (at least for two-days with exchange of water several times), centrifuged and the pellets were subjected to freeze-drying. Thus obtained AS-fractions were incubated in 2 ml of sodium *meta*-periodate, 100 mM, for 4-days at 4°C in the dark. Excess of periodate was destroyed following the addition of 400 µl of ethylene glycol. After dialysis (membrane cut-off, 1000 Da) against water; the products were reduced for 2 h in 100 mM NH_4OH containing 20 mg of $NaBH_4$. Excess reagent was removed upon repeated co-distillations with methanol. Further, Smith degradation was performed with 10% acetic acid at 100°C for 1 h and the resultant product was freeze-dried. Theoretically, these freeze-dried

samples are the sources of α-(1,3)-glucan (31); however, when we treated it with a recombinant β-(1,3)-glucanase (LamA) (32), we found β-(1,3)-oligomers accounting for 3-5% of the α-(1,3)-glucan preparation. We repeated LamA treatment until no more β-(1,3)-oligomers were released from the α-(1,3)-glucan preparation, so as to obtain pure α-(1,3)-glucan.

Preparation of α-(1,3)-glucan oligosaccharides

α-(1,3)-oligosaccharides were obtained after partial acid-hydrolysis of the α-(1,3)-glucan. In brief, 200 mg of α-(1,3)-glucan isolated from the *A. fumigatus* cell wall was stirred in a mixture of 20 ml acetic anhydride, 20 ml acetic acid and 2 ml H_2SO_4 in a closed container, at room temperature for 48 h. The reaction was arrested by adding pyridine till the mixture pH becomes neutral (approximately one volume of the reaction mixture); the contents were dialyzed against water (at least for 3-4 days with repeated water exchanges) followed by freeze-drying. The dried sample was reduced with NaOH (1 N) containing $NaBH_4$ (10 mg in 1 ml NaOH) overnight, neutralized with 2% acetic acid, centrifuged (4500 rpm, 10 min). The supernatant was collected, concentrated using rotavapor and washed-evaporated twice with methanol. The dried product obtained was washed twice with 80% ethanol; each time, after centrifugation the supernatant was discarded colleting the pellet, which was then reconstituted in water and freeze-dried. This dried sample was the source of α-(1,3)-oligo mixture, which was further dissolved in water, subjected to gel-permeation chromatography on a Sephadex G15 column, eluted with 0.25% acetic acid; the pools of fractions were collected and freeze-dried to obtain α-(1,3)-oligo mixtures of different sizes. The sizes of α-(1,3)-oligosaccharide in each pool were determined by high-performance anion exchange chromatography (HPAEC) using DIONEX as well as MALDI-analyses.

Generation and treatment of the DCs with α-(1,3)-glucan and its oligosaccharides

DCs were generated from peripheral blood monocytes using the protocol as described earlier upon obtaining the buffy bags of healthy donors from the Centre Necker-Cabanel, Etablissement Français du Sang (EFS), Paris, France (33). Institut National de la Santé et de la Recherche-EFS ethical committee permission (N°12/EFS/079) has been obtained for the use of such human samples. Immature DCs ($0.5x10^6$/ml) were cultured in the presence of GM-CSF and IL-4 alone or with α-(1,3)-glucan isolated from the *A. fumigatus* cell wall (0.5 or 1 μg/ml) for 48 h. In other experiments, DCs were also treated with 1 μg of α-(1,3)-oligo mixtures of various sizes (degree of polymerization (dp) 4-8, dp6-10, dp8-14 and dp 8-30).

To investigate the inhibitor effect of oligosaccharides (dp4-8 and dp6-10) towards α-(1,3)-glucan-mediated maturation of DCs, cells ($0.5x10^6$ cells) were cultured in GM-CSF and IL-4 alone or with 1 μg of dp4-8 or dp6-10 α-(1,3)-oligo mixture for 30 min followed by stimulation with 1 μg of α-(1,3)-glucan for 48 h.

After 48 h of incubation, cell-free culture supernatants collected were analyzed for various DC-cytokines (IL-12, IL-6, IL-8, IL-10, IL-1β, TNFα; (ELISA Ready-SET-Go, eBioscience) and DCs were processed for flow cytometry by surface staining with fluorochrome-conjugated antibodies. DCs suspended in 10% FCS/PBS buffer and fluorochrome-conjugated monoclonal antibodies against various surface molecules were added at pre-determined concentration. The samples were acquired by using LSR II (BD Biosciences) flow cytometry and the data were analyzed by BD FACS DIVA software (BD Biosciences).

For blocking experiments of PRRs, DCs were pre-incubated with MAbs to TLR-2, DC-SIGN, Dectin-1, TLR-4 (at 10 μg/$0.5x10^6$ cells/ml), isotype control antibodies or with mannan (1 mg/ml) followed by stimulation with α-(1,3)-glucan.

For blocking PD-L1 (CD274), α-(1,3)-glucan-treated DCs ($0.5x10^6$) were washed, incubated with blocking MAb to PD-L1 or isotype control antibody at a concentration of 10 μg/$0.5x10^6$ cells for 2 h and subjected for co-culture with naïve $CD4^+$ T cells.

DCs-naïve $CD4^+$ T cell co-culture

For exploring the effect of α-(1,3)-glucan-activated DCs on T cell polarization, DCs following activation were extensively washed and co-cultured with $0.1x10^6$ $CD4^+CD45RA^+CD25^-$ autologous naïve T cells at 1:10 ratio for five days in serum-free X-VIVO medium. The cell-free culture supernatants were collected and analysed for the various T cell cytokines by ELISA (IFN-γ, IL-4, and IL-17A; ELISA Ready-SET-Go, eBioscience). $CD4^+$ T cells were activated with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL, Sigma-Aldrich, France), along with GolgiStop (BD Biosciences), for 4 h. For the analysis of $CD4^+$ T cell polarization (Th1, Th2, Th17 and Treg), surface staining was performed with fluorescence-conjugated MAbs to CD4, CD127 and CD25. Cells were then fixed, permeabilized using intracellular staining kit (eBioscience), and incubated at room temperature with fluorescence-conjugated Mabs to FoxP3, IFN-γ, IL-4, and IL-17A. Samples were processed for flow cytometry.

Statistical analysis

Statistical analyses were performed by two-way parametric Student's-t-test, two-way nonparametric Mann-Whitney test or one-way ANOVA as indicated using Prism 5 software. $P<0.05$ was considered significant.

Results

A. fumigatus cell wall α-(1,3)-glucan induces maturation and activation of human DCs

Although the presence of α-(1,3)-glucan in the *A. fumigatus* cell wall is known since long time, the ability of this polysaccharide to regulate the functions of human innate immune cells was not yet explored. As education of DCs decides the fate of immune response, we first investigated the effect of α-(1,3)-glucan on DC-maturation. α-(1,3)-Glucan isolated from the mycelia induced maturation of DCs as analyzed by the phenotype. α-(1,3)-Glucan-stimulated DCs displayed significantly higher expression of CD83, co-stimulatory molecules CD80, CD86, CD40 and antigen-presenting molecule HLA-DR (Fig. 1A-B). The percentage expression of CD83 and CD86 was increased by four to five-folds (Fig. 1A) while the intensity of expression (MFI) of CD80, CD86, CD40 and HLA-DR (Fig. 1B) was enhanced nearly three-folds upon stimulation with α-(1,3)-glucan. Further, the induction of maturation of DCs by α-(1,3)-glucan was dose-dependent (Fig. 1C).

α-(1,3)-Glucan is an integral part of both conidial and mycelial morphotypes of *A. fumigatus* cell wall. When we compared the ability of α-(1,3)-glucan purified from conidia and mycelia in inducing maturation of DCs, we observed that conidial α-(1,3)-glucan was less stimulatory compared to its mycelial counterpart. While purifying α-(1,3)-glucan from the conidial cell wall, it is inevitable to separate traces of melanin; we hypothesized that melanin co-extracted with conidial α-(1,3)-glucan might have affected stimulation. To explore our hypothesis, we resorted to α-(1,3)-glucan isolated from the melanin-deficient Δ*alb1* mutant conidia (34) and found that α-(1,3)-glucan from Δ*alb1* mutant conidia was equally potent as that of mycelial α-(1,3)-glucan in inducing DC-maturation (data not shown).

DCs are known to secrete a large array of immunoregulatory cytokines when they undergo maturation process and these cytokines play a crucial role in polarizing T cell responses. Therefore, the cell-free culture supernatants of α-(1,3)-glucan-treated DCs were subjected to a detailed cytokine analysis. As expected, untreated immature DCs produced only

minimal quantities of cytokines. However, α-(1,3)-glucan induced large quantities of TNF-α, IL-6 and IL-8 (Fig. 2A-C). In addition, moderate induction of IL-1β and IL-10 was also observed (Fig. 2D-E). Of note, the secretion of IL-12p70, a key Th1-polarizing cytokine, was low (Fig. 2F).

α-(1,3)-Glucan-induced maturation of DCs is dependent on TLR2

Previous reports have demonstrated that TLRs, in particular TLR2, plays a crucial role in sensing *A. fumigatus,* in mounting anti-fungal immunity and in fungal infectivity (7, 8, 11, 35, 36). In addition, non-TLR receptors, particularly C-type lectin receptors such as Dectin-1 and DC-SIGN, were also demonstrated to recognize PAMPS of *Aspergillus* (5, 12, 14, 17, 18, 37). Therefore, we explored the PRR implicated in the signaling of DC-maturation by α-(1,3)-glucan. Blocking of lectin receptors by mannan, anti-DC-SIGN antibodies or anti-Dectin-1 antibodies as well as TLR4 blockade did not affect α-(1,3)-glucan-mediated maturation of DCs (data not shown). However, blockade of TLR-2 on DCs prior to stimulation with α-(1,3)-glucan significantly reduced the expression of co-stimulatory and antigen presenting molecules (Fig. 3).

Minimal length of α-(1,3)-glucan required for the induction of DC-maturation

The consequence of stimulation of innate immune cells by PAMP is determined by the avidity with which it recognizes receptor. The avidity of polysaccharides is generally higher as compared to soluble oligosaccharides. Therefore, we aimed at identifying the minimal length/degree of polymerization of α-(1,3)-glucan required for inducing maturation and activation of DCs. Short α-(1,3)-oligosaccharide mixtures with degree of polymerization (dp; number of monosaccharide units) of 4-8 or 6-10 failed to induce maturation of DCs as analyzed by the phenotype (Fig. 4A). The expression of various surface markers such as CD83, CD86, HLA-DR and CD40 were on par with untreated DCs. In addition, these short oligosaccharides did not induce any of the DC cytokines analyzed (Fig. 4B).

On the other hand, α-(1,3)-oligosaccharides of dp8-14/dp8-30 induced partial but significant maturation of DCs; DCs showed nearly two-fold increase in the expression of various maturation-associated molecules upon stimulation with these longer α-(1,3)-oligosaccharides (Fig. 5A). In addition, dp8-14 and dp8-30 α-(1,3)-oligosaccharides also induced low amounts of DC cytokines IL-8, IL-6, IL-1β and TNF-α (Fig. 5B). Taken together, our results show that minimal length of α-(1,3)-glucan required for the induction of DC

maturation is in the range of 11-14. Further, secretion of these cytokines was relatively higher with dp8-30, suggesting that the extent of stimulation is dependent on the α-(1,3)-glucan chain length.

Short α-(1,3)-oligosaccharides inhibit α-(1,3)-glucan-mediated maturation of DCs

The lack of induction of maturation of DCs by short oligosaccharides of α-(1,3)-glucan rises an interesting possibility that they might interfere with stimulatory functions of α-(1,3)-glucan. To confirm this, DCs were pre-treated with α-(1,3)-oligosaccharides of dp4-8 or dp6-10 followed by stimulation with α-(1,3)-glucan. In line with our hypothesis, both dp4-8 and dp6-10 significantly inhibited α-(1,3)-glucan-mediated DC-maturation (Fig. 6A). In addition, both these short oligosaccharides inhibited α-(1,3)-glucan-induced DC-cytokines (Fig. 6B).

α-(1,3)-Glucan-matured DCs polarize Treg responses

We then explored the ability of α-(1,3)-glucan-matured DCs to induce T cell polarization from naïve $CD4^+$ T cells. Analysis of various $CD4^+$ T cell subsets by intra-cellular staining either for $CD4^+$ T cell subset cytokines (IFN-γ, IL-4, IL-17A) or transcription factor (FoxP3) revealed that α-(1,3)-glucan-matured DCs polarizes predominantly $CD4^+CD25^+CD127^-$ $FoxP3^+$ Treg responses (Fig. 7A and B) whereas Th1, Th2 and Th17 responses were not significantly altered (Fig. 7C). To further validate these results, we quantified various T cell cytokines in the DC-$CD4^+$ T cell co-culture supernatants. Confirming the intra-cellular staining data (Fig 7B), we found that α-(1,3)-glucan-matured DCs induced minimal quantities of Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17A) cytokines (Fig. 7D).

α-(1,3)-Glucan-induced PD-L1 expression on the DCs dictates the balance between Treg and IFN-γ responses

We aimed at exploring the mechanism by which α-(1,3)-glucan induces Treg responses. Analysis of various co-stimulatory molecules on DCs that are implicated in Treg expansion revealed that α-(1,3)-glucan induces significantly high expression of PD-L1 (CD274) on DCs (Fig. 8A). To validate the role of PD-L1 in α-(1,3)-glucan-induced Treg responses, we employed blocking antibodies and found that PD-L1 blockade led to significant inhibition of α-(1,3)-glucan-induced Treg responses (Fig 8B and C). Of interest, secretion of protective Th1 cytokine IFN-γ was significantly enhanced upon PD-L1 blockade (Fig 8D) while, IL-17 and IL-4 were not altered (data not shown). The data thus indicate that PD-L1 on DCs dictates the

balance between Treg and IFN-γ responses induced by α-(1,3)-glucan.

Discussion

Although conidial surface rodlet-melanin layers cover underneath cell wall polysaccharides (30, 34, 38, 39), conidial swelling during germination results in the remodeling of the cell wall that exposes these polysaccharides, which in turn activate defense mechanisms of the host (40, 41). Our previous work has showed that *A. fumigatus* cell wall β-(1,3)-glucan induces Th1/Th17 responses whereas galactomannan activates Th2/Th17 responses in mice and humans (19, 31). Subsequent report demonstrated that Dectin-1-mediated signaling in murine innate cells enhances Th17 responses to *A. fumigatus* by diminishing IL-12 and IFN-γ (19, 31). We also demonstrated that GAG inhibits Th1/Th17 cytokine production in human PBMCs through the induction of interleukin-1 receptor antagonist (22). A recent study with *Candida albicans*-derived chitin demonstrated the secretion of anti-inflammatory cytokine IL-10 in human PBMCs and this IL-10 might be responsible for the lack of induction of Th1 (IFN-γ) and Th17 (IL-17) responses to chitin (42). On the other hand, cell wall chitin from *A. fumigatus* showed both pro- and anti-inflammatory properties; in presence of other PAMPs, chitin showed synergistic pro-inflammatory properties whereas there was the production of anti-inflammatory cytokine IL-1 receptor antagonist in the presence of serum immunoglobulins (43). Our present study shows that α-(1,3)-glucan, yet another major cell wall component of the *A. fumigatus*, polarizes human Treg responses. These data thus suggest that cell wall polysaccharides diversify the immune response to *A. fumigatus* by their distinct abilities to polarize specific $CD4^+$ T cell responses.

DCs are sentinels of the immune system and play a key role in the polarization of $CD4^+$ T cell responses. Interaction between PD-L1 (CD274), OX-40 ligand (OX-40L), ICOSL (CD275) on DCs and PD-1 (CD279), OX-40, ICOS on $CD4^+$ T cells have been shown to induce Tregs (44-47). We found that α-(1,3)-glucan did not induce OX-40L and ICOSL on the DCs, ruling out the involvement of these co-stimulatory molecules in α-(1,3)-glucan-mediated Treg polarization. However, α-(1,3)-glucan induced high expression of PD-L1 on DCs, the interaction of which with PD-1 promotes Treg responses (44) and efficiently suppresses Th1 response (48). Further, interaction of PD-L1 with PD-1 on T cells leads to the activation of SHP1/2 that suppresses STAT1, resulting in the abrogation of IFN-γ production. Thus, low level induction of IL-12p70 in association with high PD-L1 expression on the DCs explains the low Th1 polarization by 'α-(1,3)-glucan-educated' DCs. However, Bittencourt et al.

observed secretion of IL-12 in murine DCs when exposed to *Pseudallescheria boydii* cell wall-derived α-glucan (49). This could be attributed to the fact that *P. boydii*-derived α-glucan contains linear α-(1,4)-linkages with side-chains substituted at carbon-6 position of glucose, whereas *A. fumigatus*-derived glucan is α-(1,3)-glucan with an average of 1% intra-chain α-(1,4)-linked glucose residues. This enforces the fact that the linkage pattern of a polysaccharide plays a crucial role in the kind of immune stimulatory mechanism.

Our previous collaborative work has reported that α-(1,3)-glucan when injected to mice in combination with CpG or upon adoptive transfer of α-(1,3)-glucan–pulsed $CD11c^+$ splenic DC, conferred protection upon challenge with *A. fumigatus* (31). This protection was associated with induction of Th1 (IFN-γ) and Treg responses. However, we found that α-(1,3)-glucan-pulsed human DCs polarized predominantly Treg responses. The IFN-γ production was quite low; clear Th1 response was observed only upon PD-L1 blockade on DCs. The discrepancy in the results might be attributed to various factors including vaccination model used in *in vivo* murine experiments, differences in experimental system, differences in the expression pattern of PD-L1 and other PRR, or due to the protocol used to isolate α-(1,3)-glucan during previous work. Upon encounter with α-(1,3)-glucan *in vivo*, the final immune response will depend not only on the relative degree of stimulation of particular innate immune cells but also on their diversity, cooperation of other innate cells, non-immune cells and PRRs, and the cellular localization.

Studies have demonstrated that among TLRs, innate recognition of *A. fumigatus* is mediated by TLR4 and TLR2 (7, 8, 10, 11, 35, 36, 50). Thus, TLR2 occupies a unique place in *A. fumigatus* pathogenesis, as it appears to be one of the major PRRs that interact with this pathogen. However, a specific receptor on the immune cells that recognizes *A. fumigatus* cell wall α-(1,3)-glucan has not been identified so far, though this polysaccharide contributes for the major amorphous part of the cell wall. Our data indicate that TLR2 is implicated in the cross-talk between α-(1,3)-glucan and the human DCs. There was significant reduction in the expression of co-stimulatory molecules on DCs upon blockade of TLR2 prior to stimulation with α-(1,3)-glucan. In line with our observation, *P. boydii* cell wall-derived α-glucan was demonstrated to induce cytokine secretion from mouse peritoneal macrophages and bone marrow-derived DCs in a TLR2-dependent mechanism (49); the cytokine secretion was abolished in the macrophages and DCs from TLR2-knockout mice. Further, Chai et al. observed internalization of TLR2 by *A. fumigatus* conidia (10). This leads to the hypothesis

that, though α-(1,3)-glucan is not an integral component of the conidial surface structure, conidial phagocytosis followed by its attempt to undergo germination inside the phagosome and exposing the cell wall polysaccharides on to the surface as well as conidia-induced TLR2 internalization may be interlinked, which has to be validated.

By using functional assays where DC-maturation and release of cytokines as read-outs, we show that α-(1,3)-oligosaccharides having dp4-8 and 6-10 are not stimulatory whereas, increase in the chain (dp8-14 and 8-30) enables them to stimulate DCs. The data thus suggest that minimum α-(1,3)-oligosaccharides chain-length required to stimulate DCs is in the range of dp10-14. Importantly, the short α-(1,3)-oligosaccharides (dp4-8 and 6-10) efficiently blocked DC-stimulatory ability of α-(1,3)-glucan. These data fits well with the 'fibril hypothesis' which proposes that soluble short oligosaccharides recognize a single PRR without inducing an immune response, whereas the fibrillar polysaccharides bind to several PRR molecules leading to increased avidity, triggering of signaling events and activation of the immune response (16). Based on our present observation, we further suggest that short oligosaccharides could be used as therapeutic molecules to reduce fungal-associated inflammation and to inhibit non-protective immune responses mediated by a given polysaccharide (for example galactosaminogalactan) (21, 22, 51).

In *A. fumigatus*, α-(1,3)-glucan biosynthesis is regulated by three genes, *AGS1*, *AGS2* and *AGS3*. Based on our recent data from triple mutants that lack all three *AGS* genes, it was concluded that α-(1,3)-glucan is dispensable for growth and germination of *A. fumigatus* (29, 52). However, α-(1,3)-glucan is found to be important for maintaining the integrity of the *A. fumigatus* cell wall, as triple *AGS* deletion mutant conidial cell wall was leaky, containing an amorphous protein-layer covering the surface (29). Because of the surface modifications, despite being dormant, these mutant Δ*ags* conidia induced secretion of inflammatory cytokines from the macrophages. Thus, in addition to its role as PAMP that activates innate immune cells including DCs, α-(1,3)-glucan is also important in maintaining the integrity of conidial surface and in determining the immunogenicity of dormant conidia. In addition, *in vivo*, α-(1,3)-glucans are identified in the biofilm matrix of the pulmonary aspergilloma, where hyphae remain strongly aggregated. However, during invasive aspergillosis where hyphae were disseminated in the lungs of the patients, α-(1,3)-glucans are seen in the inner layer of the cell wall (53). Analysis of *A. fumigatus* cell wall architecture and composition *in vivo*, particularly in the immunodeficient individuals, should shed light on the localization of α-(1,3)-glucan in

the conidia during germination and its interaction with innate immune cells.

Several pathogens evade the protective immune responses by exploiting PD-L1-PD-1 pathway (54). Our data indicate that fungal pathogens such as *A. fumigatus* could also exploit this pathway for the immune evasion and persistence. In addition to causing T cell exhaustion and anergy, PD-L1-PD-1 pathway also inhibits IFN-γ responses (55, 56). Of note, PD-L1-PD-1 pathway blockade leads to enhanced IFN-γ response (55-58) and inhibition of Treg expansion (46, 59). Restoration of protective T cell response by blocking PD-1–PD-L1 axis has emerged as an important therapeutic intervention in various forms of cancer including advanced stages of melanoma and non-small cell lung cancer (60, 61). In line with these reports, blockade of PD-L1 on 'α-(1,3)-glucan-educated' DCs led to enhanced secretion of IFN-γ without modulation of Th2 and Th17 responses. Th1 response associated with an enhanced production of IFN-γ plays a major protective role in curtailing *A. fumigatus* invasion (6, 62, 63). Importantly, patients with invasive aspergillosis secrete low levels of IFN-γ and administration of IFN-γ, adjunct to anti-fungal therapy, was associated with significant clinical improvements, contrast to only anti-fungal therapy with progression in the disease condition (64, 65). Taken together, these results demonstrate the mechanism by which cell wall components of *A. fumigatus* such as α-(1,3)-glucan could dampen protective immune response against *Aspergillus* infection. Furthermore, our results also provide a rational for exploiting immunotherapeutic approaches that target PD-1–PD-L1 co-stimulatory axis to restore effector T cell response to *A. fumigatus*.

Figure Legends

FIGURE 1. α-(1,3)-Glucan from *A. fumigatus* mycelial cell wall induces maturation of human DCs. (**A and B**) Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. Phenotypic analysis of DCs was performed by flow cytometry. (**A**) Percent-cells positive for the expression of CD83 and CD86. (**B**) The mean fluorescence intensities (MFI) of expression of CD80, CD86, HLA-DR and CD40. Data are mean±SEM from four independent experiments; **, P <0.01; *** P <0.001 as determined by two-way Student's -t-test. (**C**) α-(1,3)-Glucan induces maturation of DCs in a dose-dependent manner. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or α-(1,3)-glucan (0.5 and 1 μg/0.5x10^6 cells/ml) for 48 h. Percent-cells positive for the expression of CD83 and CD86 are presented (n=3, mean±SEM). *, P <0.05; **, P <0.01 as analyzed by one-way ANOVA test.

FIGURE 2. *A. fumigatus* mycelial cell wall α-(1,3)-glucan stimulates cytokine secretion from human DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. The amounts (mean ± SEM, n=4) of (**A**) TNFα (**B**) IL-8, (**C**) IL-6, (**D**) IL-1β, (**E**) IL-10 and (**F**) IL-12 in the cell-free culture supernatants were analysed. * P <0.05; **, P <0.01 as determined by two-way Student's-t-test.

FIGURE 3. Induction of maturation of dendritic cells by α-(1,3)-glucan is dependent in part on TLR2. (**A**) DCs were cultured in GM-CSF and IL-4 alone (Ctr) or in the presence of anti-TLR2 blocking monoclonal antibodies or isotype control antibodies. After 1 h blocking, DCs were stimulated with α-(1,3)-glucan. The expression of CD86, HLA-DR and CD40 (MFI) are presented. Data are mean ± SEM from four independent experiments. *, P <0.05 as analyzed by one-way ANOVA test.

FIGURE 4. Short α-(1,3)-oligosaccharides (dp4-8 and dp6-10) lack the capacity to induce maturation of DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-oligosaccharides (dp4-8 or dp6-10) or α-(1,3)-glucan for 48 h. (**A**) Phenotypic analysis of DCs was performed by flow cytometry. The expression of CD83, CD86 (% positive cells), HLA-DR and CD40 (MFI) are presented. (**B**) The amount of IL-6, IL-8, IL-1β and TNFα in the cell-free culture supernatants. Data are mean±SEM from three independent experiments; *, P < 0.05; **, P < 0.01; *** P < 0.001; ns, not significant as determined by one-way ANOVA test.

FIGURE 5. Longer α-(1,3)-oligosaccharides (dp8-14 and dp8-30) induce maturation of DCs and cytokine secretion. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-oligosaccharides dp8-14 or dp8-30 for 48 h. (**A**) The expression of CD83, CD86 (% positive cells), CD40 and HLA-DR (MFI) are presented. (**B**) The amount of IL-6, IL-8, TNF-α and IL-1β in the cell-free culture supernatants. Data are mean±SEM from five independent experiments; *, P < 0.05; ns, not significant as determined by one-way ANOVA test.

FIGURE 6. Short α-(1,3)-oligosaccharides (dp4-8 and dp6-10) inhibit α-(1,3)-glucan-mediated maturation of DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-oligosaccharides (dp4-8 or dp6-10) for 30 min followed by stimulation with α-(1,3)-glucan for 48 h. DCs stimulated with α-(1,3)-glucan alone served as control. (**A**) Phenotypic analysis of DCs was performed by flow cytometry. The expression of CD83, CD86 (% positive cells), HLA-DR and CD40 (MFI) are presented. (**B**) The amount of IL-6, IL-8, IL-1β and TNFα

in the cell-free culture supernatants. Data are mean±SEM from five to six independent experiments; *, $P < 0.05$; *** $P < 0.001$; ns, not significant as determined by one-way ANOVA test.

FIGURE 7. α-(1,3)-Glucan-matured DCs polarize predominantly Treg responses. Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. DCs were washed extensively and co-cultured with autologous naïve $CD4^+$ T cells at 1:10 ratio in serum-free X-VIVO medium for five days. The $CD4^+$ T cells were analyzed for the polarization of various T cell subsets. (**A)** Representative dot-plot of $CD4^+CD25^+CD127^-$ $FoxP3^+$ regulatory T cells. **(B**) Treg response (Mean ± SEM, n= 6 donors) induced by 'α-(1,3)-glucan-educated' DCs. (**C**) Polarization of Th1, Th2 and Th17 responses as analyzed by intracellular staining for IFN-γ, IL-4, and IL-17A respectively by flow cytometry. Mean ± SEM of data from four donors. (**D**) Amount of secretion of $CD4^+$ T cell cytokines IFN-γ, IL-4 and IL-17A in the cell-free culture supernatants of DC–T cell co-cultures. Mean ± SEM of data from six donors; *, $P < 0.05$; ns, not significant as determined two-way nonparametric Mann-Whitney test.

FIGURE 8. α-(1,3)-Glucan-induced PD-L1 expression on the DCs dictates the balance between Treg and IFN-γ responses. Monocyte-derived DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with α-(1,3)-glucan from *A. fumigatus* mycelia for 48 h. **(A)** Expression of PD-L1 (CD274) on DCs (Mean±SEM, n= 6 donors) as analyzed by flow cytometry. *, $P < 0.05$; as determined by two-way nonparametric Mann-Whitney test. **(B, C and D)** DCs were washed extensively and incubated with blocking MAb to PD-L1 or isotype control antibody and subjected for co-culture with autologous naïve $CD4^+$ T cells at 1:10 ratio. **(B and C)** Modulation of $CD4^+CD25^+CD127^-FoxP3^+$ Tregs in DC:T cell co-cultures incubated with anti-PD-L1 or isotype control MAbs. Representative dot-plot and Mean±SEM of data from six donors are presented **(D)** Modulation of IFN-γ (Mean±SEM, n= 6 donors) secretion in DC-T cell co-cultures upon PD-L1 blockade. *, $P < 0.05$; ** $P < 0.01$; ns, not significant as determined by one-way ANOVA test.

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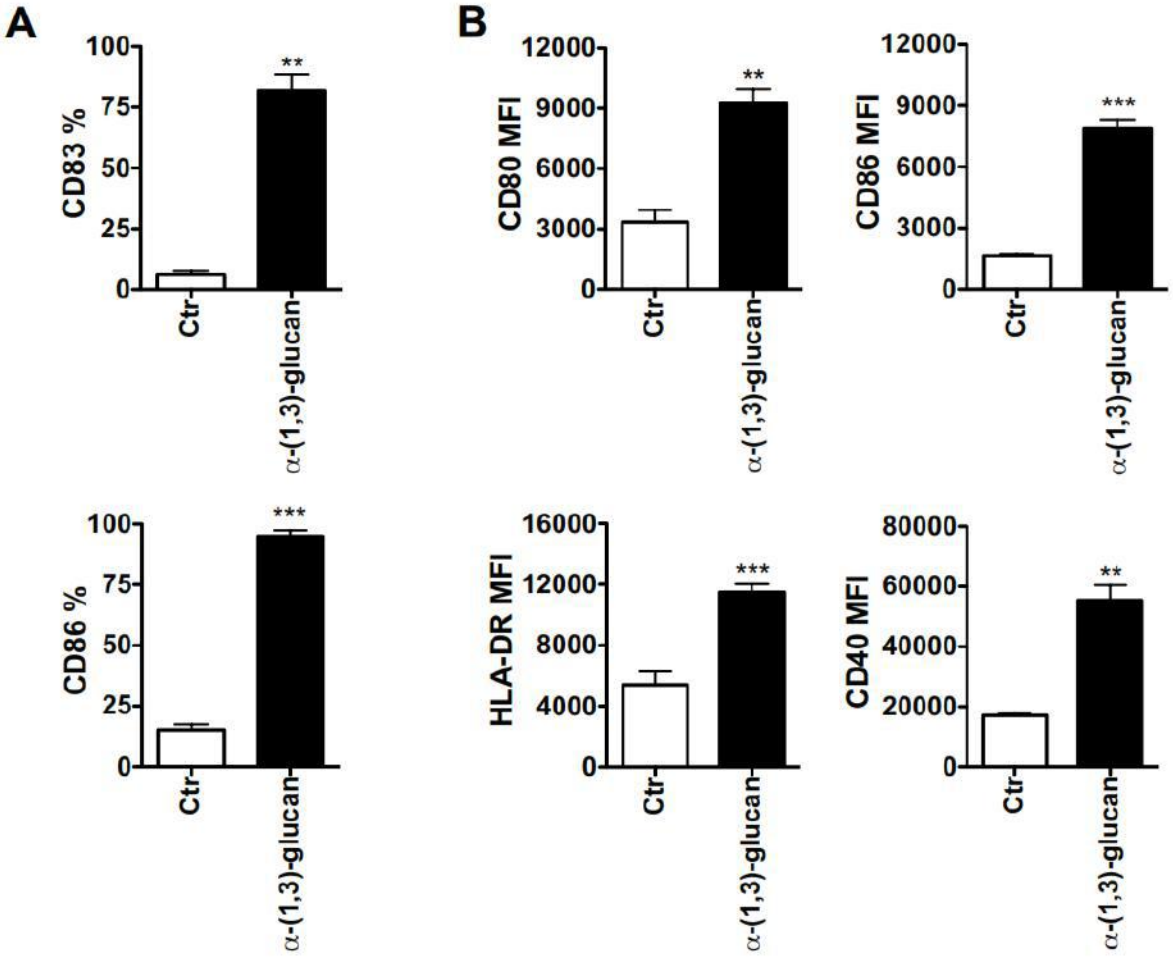
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CD83 %
CD86 %
B
CD80 MFI
CD86 MFI
HLA-DR MFI
CD40 MFI
Ctr
α-(1,3)-glucan

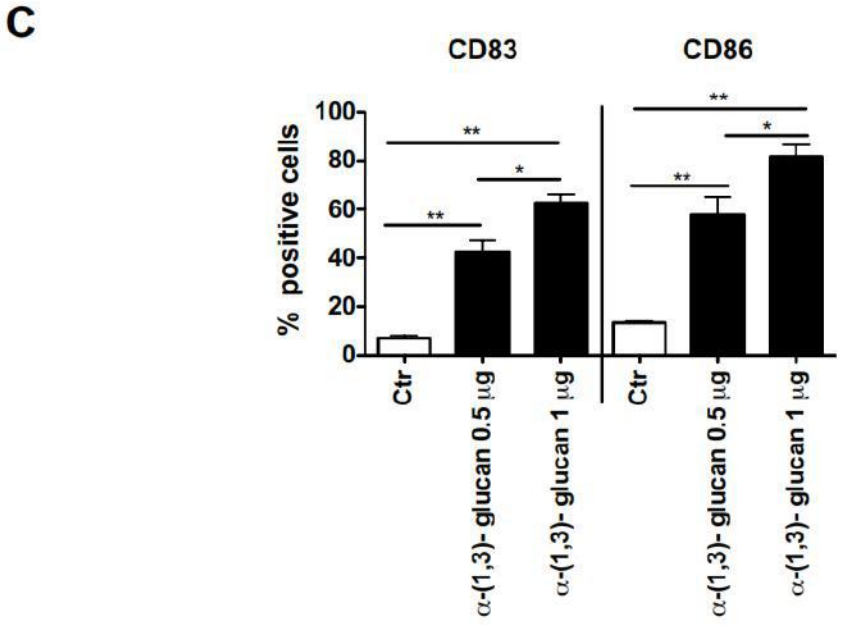

C
CD83
CD86
% positive cells
Ctr
α-(1,3)- glucan 0.5 μg
α-(1,3)- glucan 1 μg

Figure 1

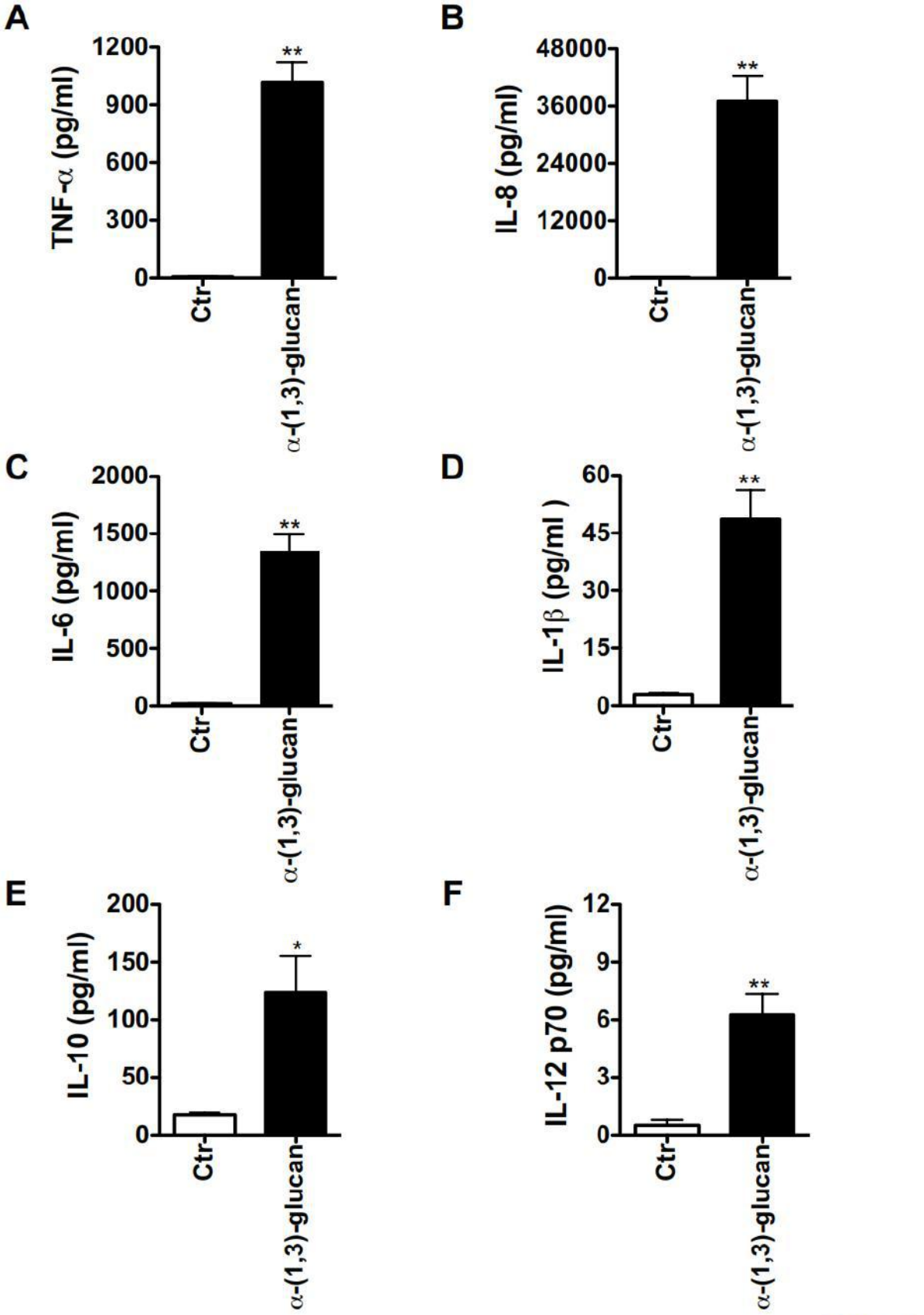
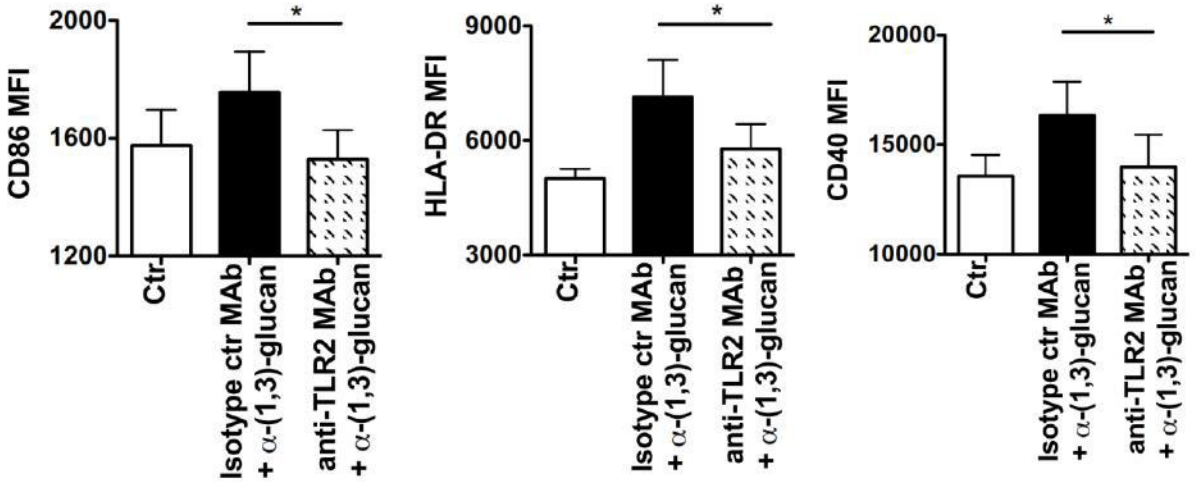

A
TNF-α (pg/ml)
1200
900
600
300
0
**
Ctr
α-(1,3)-glucan
B
IL-8 (pg/ml)
48000
36000
24000
12000
0
**
Ctr
α-(1,3)-glucan
C
IL-6 (pg/ml)
2000
1500
1000
500
0
**
Ctr
α-(1,3)-glucan
D
IL-1β (pg/ml)
60
45
30
15
0
**
Ctr
α-(1,3)-glucan
E
IL-10 (pg/ml)
200
150
100
50
0
*
Ctr
α-(1,3)-glucan
F
IL-12 p70 (pg/ml)
12
9
6
3
0
**
Ctr
α-(1,3)-glucan

Figure 2


CD86 MFI
2000
1600
1200
*
Ctr
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anti-TLR2 MAb + α-(1,3)-glucan
HLA-DR MFI
9000
6000
3000
*
Ctr
Isotype ctr MAb + α-(1,3)-glucan
anti-TLR2 MAb + α-(1,3)-glucan
CD40 MFI
20000
15000
10000
*
Ctr
Isotype ctr MAb + α-(1,3)-glucan
anti-TLR2 MAb + α-(1,3)-glucan

Figure 3

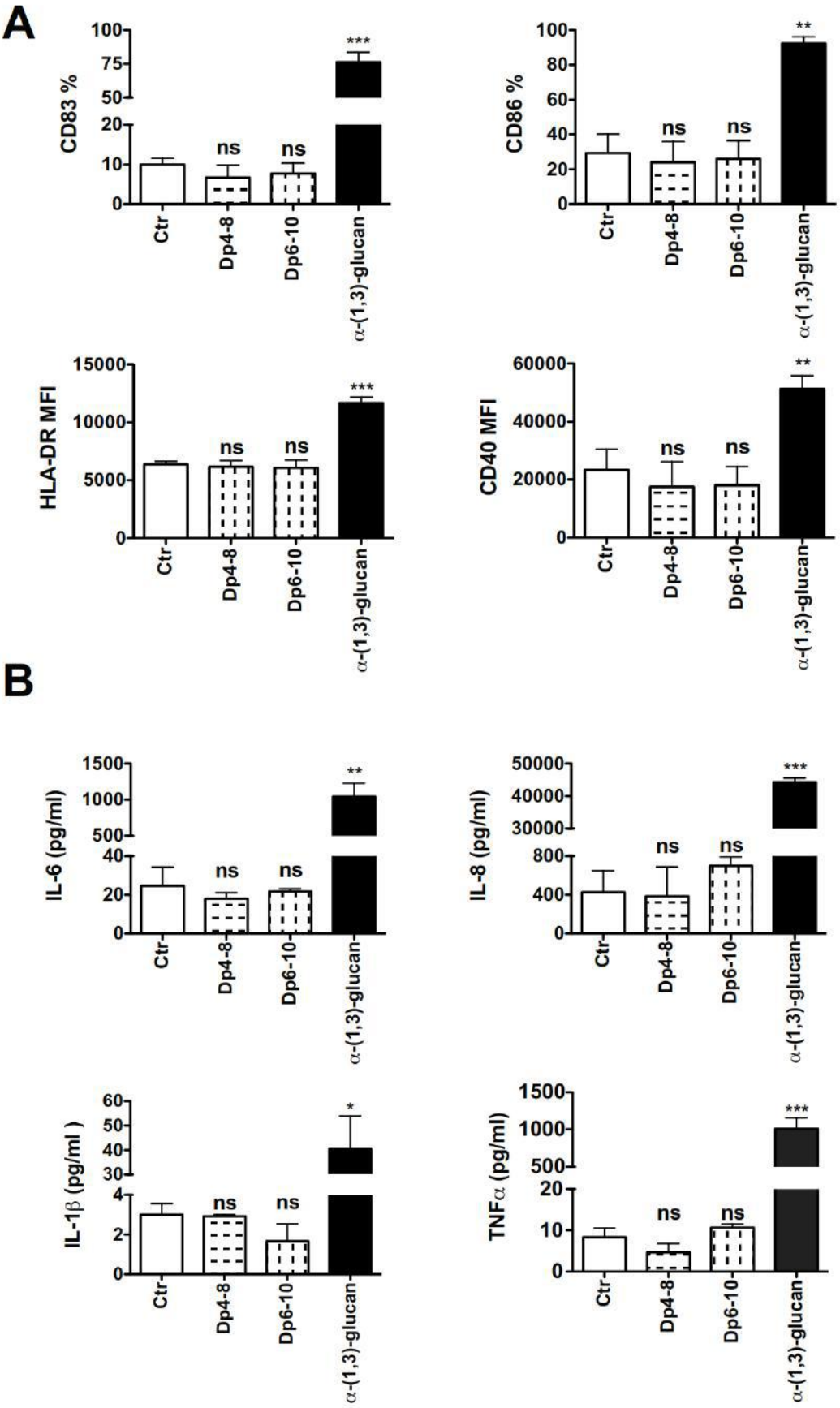
A
CD83 %
CD86 %
HLA-DR MFI
CD40 MFI
B
IL-6 (pg/ml)
IL-8 (pg/ml)
IL-1β (pg/ml)
TNFα (pg/ml)
Ctr
Dp4-8
Dp6-10
α-(1,3)-glucan
ns
*
**
***

Figure 4

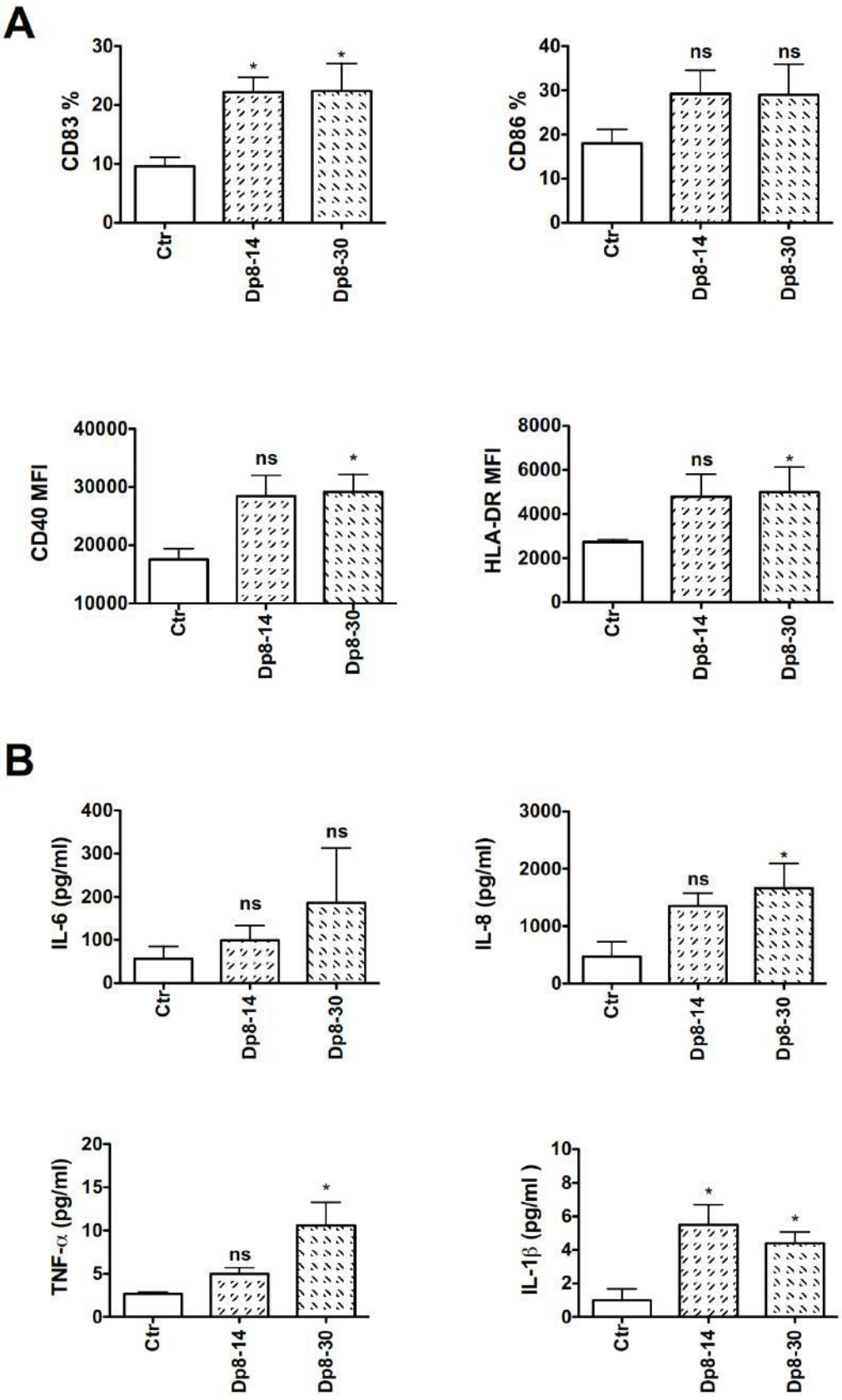
A
CD83 %
30
20
10
0
*
*
Ctr
Dp8-14
Dp8-30
CD86 %
40
30
20
10
0
ns
ns
Ctr
Dp8-14
Dp8-30
CD40 MFI
40000
30000
20000
10000
ns
*
Ctr
Dp8-14
Dp8-30
HLA-DR MFI
8000
6000
4000
2000
0
ns
*
Ctr
Dp8-14
Dp8-30
B
IL-6 (pg/ml)
400
300
200
100
0
ns
ns
Ctr
Dp8-14
Dp8-30
IL-8 (pg/ml)
3000
2000
1000
0
ns
*
Ctr
Dp8-14
Dp8-30
TNF-α (pg/ml)
20
15
10
5
0
ns
*
Ctr
Dp8-14
Dp8-30
IL-1β (pg/ml)
10
8
6
4
2
0
*
*
Ctr
Dp8-14
Dp8-30

Figure 5

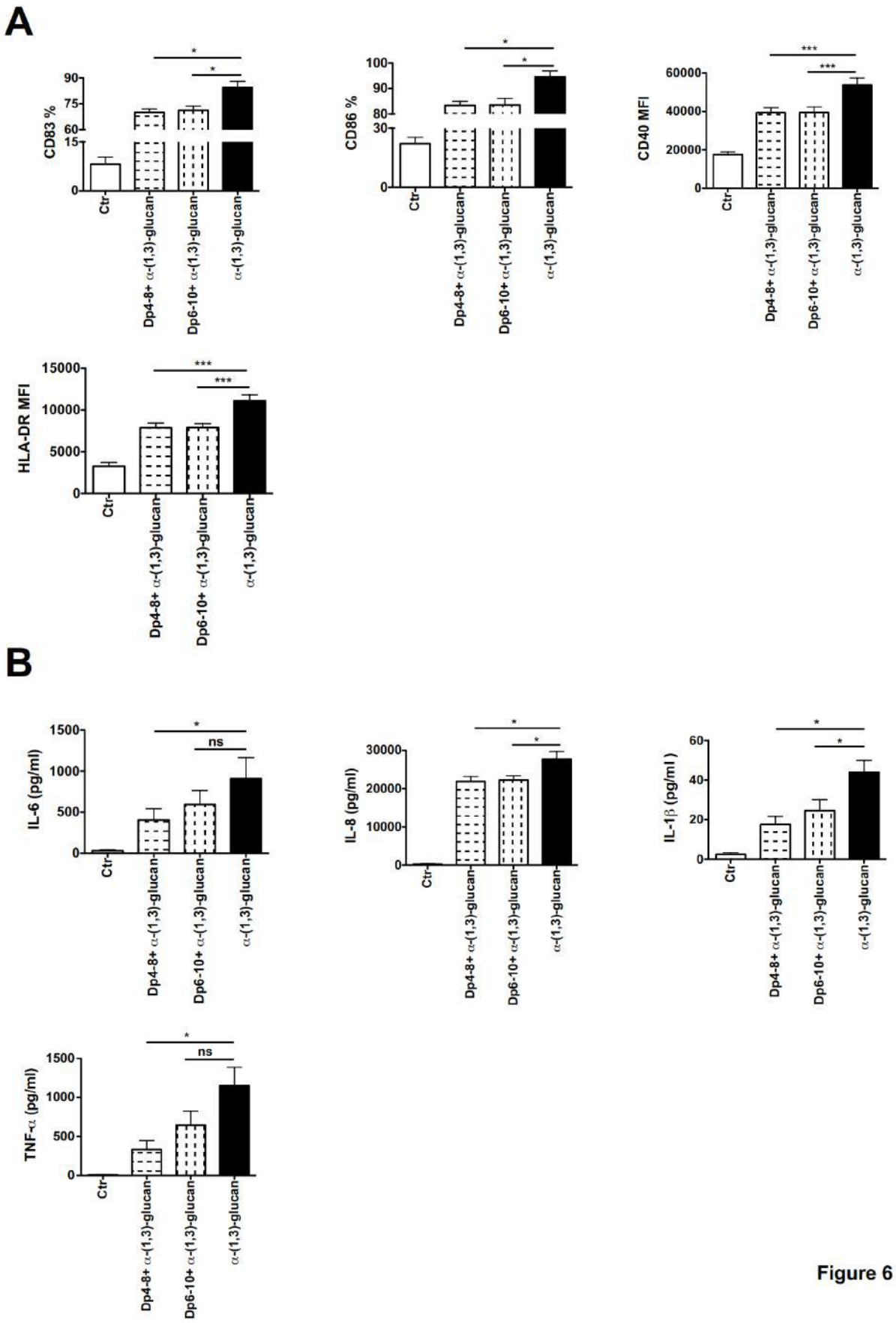
A
CD83 %
CD86 %
CD40 MFI
HLA-DR MFI
Ctr
Dp4-8+ α-(1,3)-glucan
Dp6-10+ α-(1,3)-glucan
α-(1,3)-glucan
B
IL-6 (pg/ml)
IL-8 (pg/ml)
IL-1β (pg/ml)
TNF-α (pg/ml)
ns

Figure 6

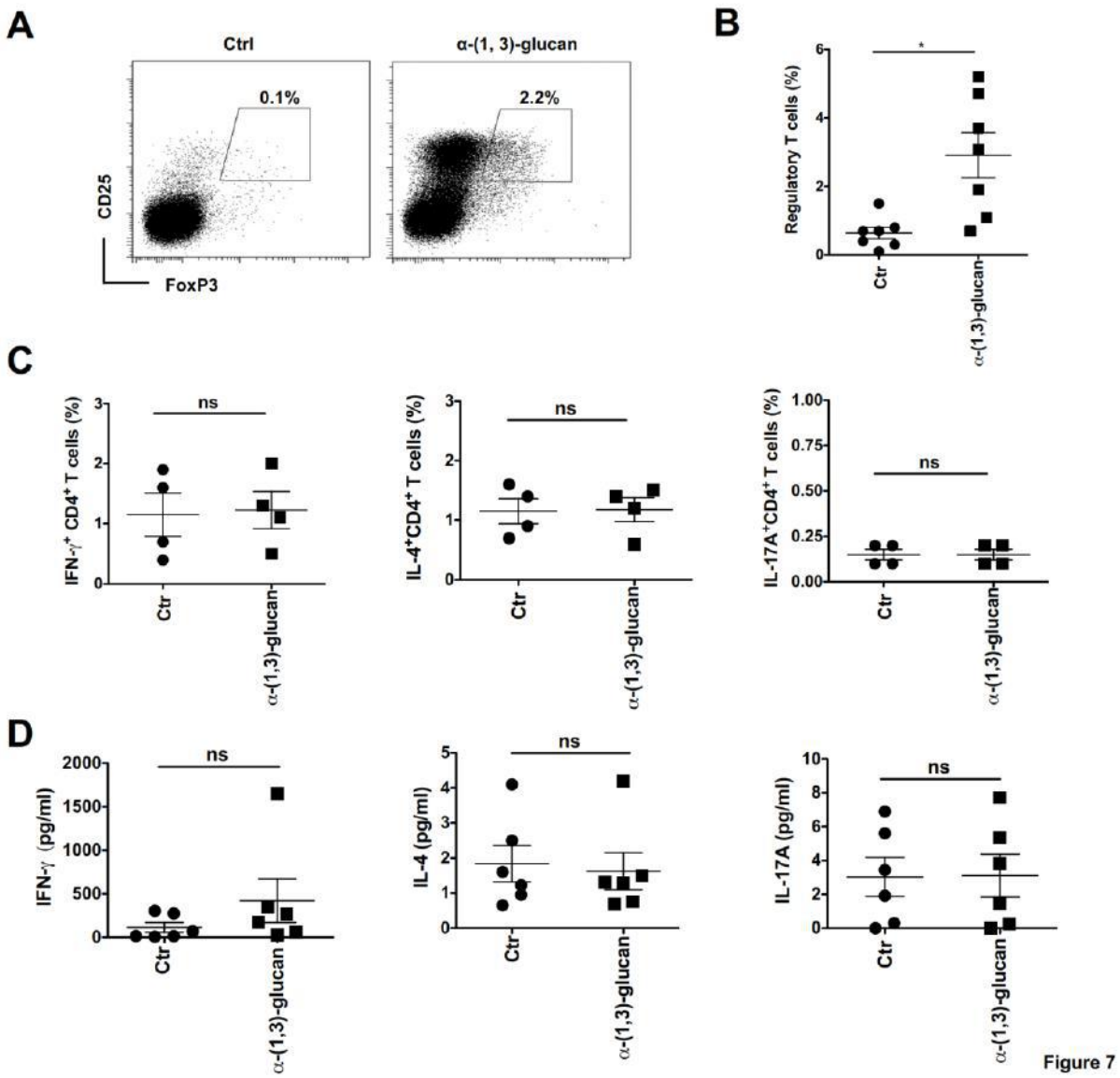
A
Ctrl
α-(1, 3)-glucan
0.1%
2.2%
CD25
FoxP3
B
Regulatory T cells (%)
Ctr
α-(1,3)-glucan
C
IFN-γ+ CD4+ T cells (%)
IL-4+CD4+ T cells (%)
IL-17A+CD4+ T cells (%)
ns
D
IFN-γ (pg/ml)
IL-4 (pg/ml)
IL-17A (pg/ml)

Figure 7

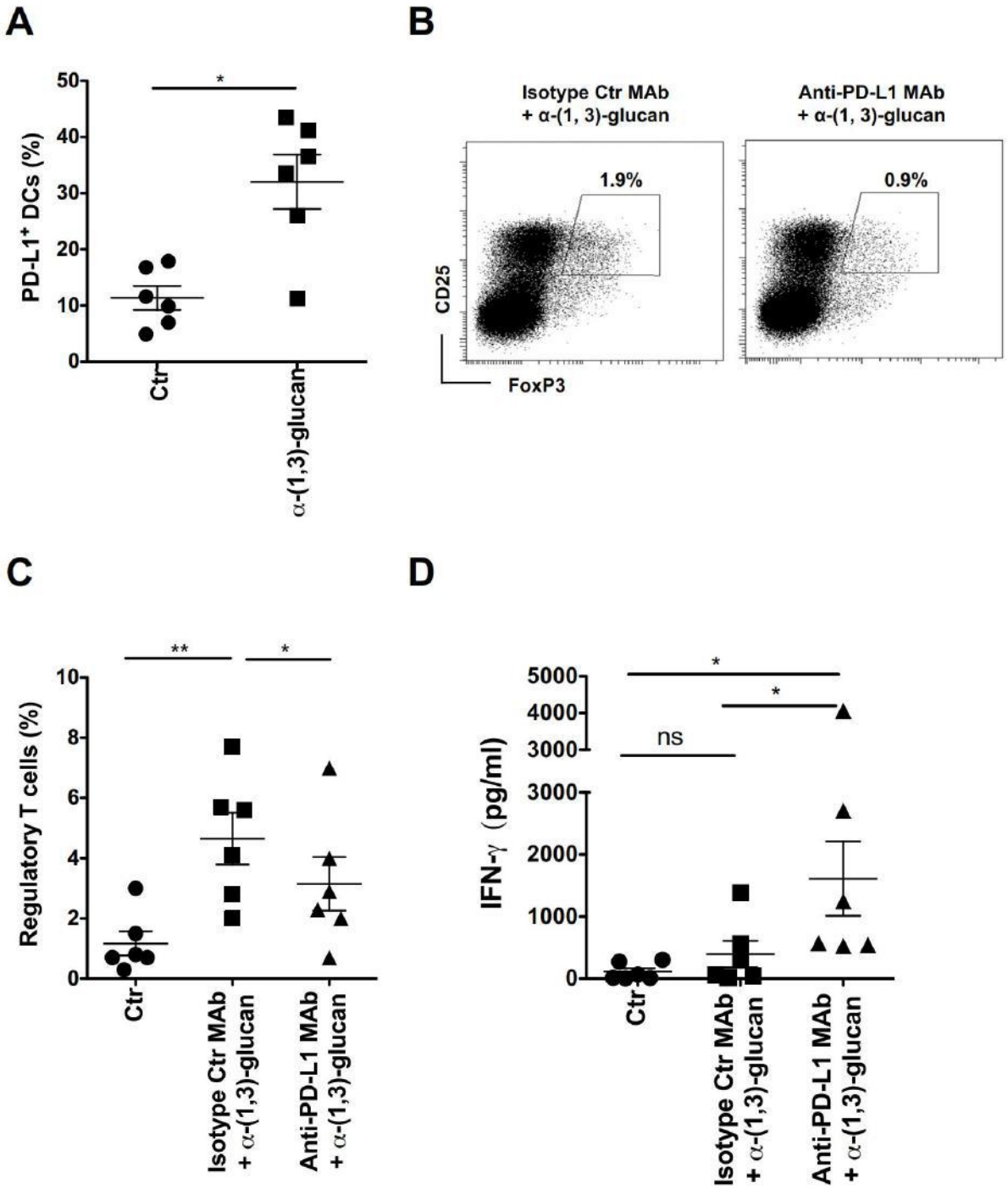
A
B
C
D
PD-L1+ DCs (%)
Ctr
α-(1,3)-glucan
Isotype Ctr MAb
+ α-(1, 3)-glucan
Anti-PD-L1 MAb
+ α-(1, 3)-glucan
1.9%
0.9%
CD25
FoxP3
Regulatory T cells (%)
Isotype Ctr MAb
+ α-(1,3)-glucan
Anti-PD-L1 MAb
+ α-(1,3)-glucan
IFN-γ (pg/ml)
ns

Figure 8

Mycobacteria-responsive sonic hedgehog signaling mediates programmed death-ligand 1- and prostaglandin E2-induced regulatory T cell expansion.

(Article II)

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Mycobacteria-responsive sonic hedgehog signaling mediates programmed death-ligand 1- and prostaglandin E_2-induced regulatory T cell expansion

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$CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) are exploited by mycobacteria to subvert the protective host immune responses. The Treg expansion in the periphery requires signaling by professional antigen presenting cells and in particularly dendritic cells (DC). However, precise molecular mechanisms by which mycobacteria instruct Treg expansion via DCs are not established. Here we demonstrate that mycobacteria-responsive sonic hedgehog (SHH) signaling in human DCs leads to programmed death ligand-1 (PD-L1) expression and cyclooxygenase (COX)-2-catalyzed prostaglandin E_2 (PGE_2) that orchestrate mycobacterial infection-induced expansion of Tregs. While SHH-responsive transcription factor GLI1 directly arbitrated COX-2 transcription, specific microRNAs, miR-324-5p and miR-338-5p, which target PD-L1 were downregulated by SHH signaling. Further, counter-regulatory roles of SHH and NOTCH1 signaling during mycobacterial-infection of human DCs was also evident. Together, our results establish that *Mycobacterium* directs a fine-balance of host signaling pathways and molecular regulators in human DCs to expand Tregs that favour immune evasion of the pathogen.

$CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) are one of the subsets of $CD4^+$ T helper cells that are necessary for immune homeostasis. Apart from their roles in maintaining peripheral tolerance, Tregs have been heavily implicated for regulating immune responses against the invading pathogens[1–3]. Exhibiting contrast functions, Tregs can not only suppress protective immune responses and the collateral damage caused due the excessive inflammation during infections, but also provide suitable niche for facilitating the persistence of chronic infections such as tuberculosis[4–6]. Thus, induction and expansion of Tregs mark as one of the immune evasion strategies of mycobacteria for their survival in the host.

During mycobacterial infections, accumulation and proliferation of Tregs at the site of infection contributes to inhibition of bacterial clearance[5,7] as well as inhibition of antigen-specific protective responses exhibited by γδ T cells[8]. Further, patients with active tuberculosis have increased population of circulating Tregs that suppress IFN-γ production by Th1 cells[9]. Correspondingly, *ex vivo* depletion of Tregs from the PBMCs alleviates the IFN-γ production in response to mycobacterial antigens[10]. While *in vivo* depletion of $CD25^+$ T cells in mice enhanced the IFN-γ production, adoptive transfer of $CD25^+$ T cells to mice infected with *M. tuberculosis* facilitates bacterial survival[11]. Thus, understanding the mechanisms that define such Treg-mediated survival strategies of mycobacteria is important.

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Investigations in both murine and human models of tuberculosis have identified several mechanisms of Treg generation and expansion[12,13]. Of current interest, studies have highlighted the roles for PD-L1 (B7-H1/CD274) and COX-2-catalyzed PGE_2 during mycobacteria-induced Treg induction and expansion. While PD-L1 deficient mice were increasingly sensitive to tuberculosis infection[14], studies on human DCs showed that infection-induced PD-L1 was essential for expansion of Tregs[15,16]. Though PD-L1 KO mice exhibited elevated $CD4^+$ T and $CD8^+$ T cell responses, PD-1 expression was higher in $CD4^+$ T cells in the PD-L1 KO mice, suggesting a possible suppression of PD-1 by PD-L1. Further, possibly due to chronic activation of immune cells and inflammation, PD-L1 KO mice exhibited increased mycobacterial CFUs in lung and death of mice[14]. Likewise, PGE_2-responsive human Treg expansion was found during mycobacterial infection[17]. However, the mechanisms that mediate the expression of the molecules like PD-L1 and COX-2 in DCs are not established.

In this context, it is well constituted that mycobacterial infection of the cells instigates a plethora of signaling pathways that ultimately regulate the immune mediators to determine cell-fate decisions and outcome/s of the infection. Previous investigations from our laboratory implicates the roles for SHH, WNT, NOTCH1 and PI3K signaling pathways in modulating macrophage[18–21] and DC[22–24] responses. Further, NOTCH[25,26], WNT[27,28], and PI3K[29,30] pathways were entailed to regulate the DC functions. Thus, we explored the roles for these signaling pathways in modulating the mycobacteria-induced Treg expansion and functions.

Here we demonstrate that infection-responsive activation of SHH-PI3K-mTOR-NF-κB signaling in human DCs was necessary for *M. bovis* BCG-induced Treg expansion. On the other hand, NOTCH signaling hindered the ability of the infected DCs to expand Tregs while the contribution of WNT signaling was not evident. Although no apparent influence of SHH and NOTCH1 signaling on DC phenotype in terms of the maturation markers HLA-DR, CD40, CD83, CD80 and CD86 was observed, pro-inflammatory cytokines such as TNF-α, IL-2, IL-1β and IL-6 were moderately NOTCH1-responsive and suppressed by SHH signaling. Further, experiments utilizing pharmacological inhibitors and conventional siRNAs indicated that both PD-L1 and COX-2/PGE_2 were induced in DCs upon stimulation with *M. bovis* BCG and *M. tuberculosis* and were regulated by SHH signaling. While SHH-responsive transcription factor, GLI1 arbitrated COX-2 expression, mycobacteria-stimulated SHH signaling was found to suppress miR-324-5p and miR-338-5p, bonafide miRNAs that target PD-L1, to aid increased expression of PD-L1 and Treg expansion. Interestingly, inhibition of NOTCH1 signaling resulted in elevated expression of infection-induced PD-L1 whereas inhibition of SHH signaling showed increased transcripts of *JAG2* and NICD, markers for NOTCH activation. These results establish the mechanism of Treg expansion during mycobacterial infections, a testimony of its survival capabilities in the host.

Results

SHH and NOTCH signaling regulate *M. bovis* BCG-induced Treg expansion. To investigate the molecular circuitry regulating mycobacteria-mediated Treg expansion, role for signaling pathways like NOTCH, WNT and SHH were assessed. Twenty-four hours post-infection with *M. bovis* BCG, DCs were washed and co-cultured with autologous $CD4^+$ T cells for 5 days to analyze the expansion of Treg population. As shown in Fig. 1a,b, *M. bovis* BCG-infected DCs induced significant expansion of $CD4^+CD25^+FoxP3^+$ Treg cells. However, DCs pretreated with cyclopamine (SHH pathway inhibitor) failed to induce Treg expansion. Further, treatment of DCs with GSI (NOTCH signaling activation inhibitor) enhanced their ability to expand Tregs. Interestingly, no significant difference was observed on perturbation of WNT pathway using IWP-2 (a WNT secretion inhibitor). These results suggest that SHH signaling positively regulates *M. bovis* BCG-mediated $CD4^+CD25^+FoxP3^+$ Treg expansion and on the contrary, NOTCH signaling was found to exhibit negative regulation. Importantly, no definite roles for SHH and NOTCH signaling was found in mediating Th1 cell cytokine IFN-γ on *M. bovis* BCG infection (Fig. 1c,d). However, NOTCH signaling was required for infection-induced IL-2 (Fig. 1d). These data thus suggest selective role of SHH and NOTCH signaling pathways in modulating human Treg responses without disturbing IFN-γ responses. In line with these observations, perturbation of SHH and NOTCH signaling did not directly modulate either *M. bovis* BCG-induced DC maturation (Fig. 2a), or infection-stimulated secretion DC cytokines like TNF-α and IL-6 (Fig. 2b). Interestingly, infection-induced Th1-polarizing IL-12p70 was found to be SHH and NOTCH signaling dependent. Together, results suggest that while SHH signaling was found to be essential for *M. bovis* BCG-induced $CD4^+CD25^+FoxP3^+$ Treg expansion, NOTCH signaling suppress Treg expansion.

***M. bovis* BCG-responsive SHH signaling is PI3K-mTOR-NF-κB pathway dependent.** To further understand the molecular mechanism involved in *M. bovis* BCG-mediated SHH signaling, the activation status of SHH signaling in the DCs was assessed. Stimulation of DCs with *M. bovis* BCG or *M. tuberculosis* induced significant activation of SHH signaling pathway (Fig. 3a,b). Activation of canonical SHH pathways is marked by elevated transcripts of *SHH*, *GLI1* and *PTCH1* and increased levels of SHH, GLI1, pGSK-3β (Ser9) and decreased NUMB expression. Mycobacteria-stimulated activation of SHH signaling was found to be dependent on PI3K-mTOR-NF-κB pathway as pretreatment of DCs with specific inhibitors of PI3K (LY294002), mTOR (Rapamycin) and NF-κB (BAY 11-7085) led to significant reduction of *M. bovis* BCG-induced SHH signaling (Fig. 3c,d). Corroborating these results, DCs pretreated with mTOR or NF-κB specific inhibitors failed to expand $CD4^+CD25^+FoxP3^+$ Treg cells (Fig. 3e). However, PI3K-specific inhibitor had no effect on *M. bovis* BCG-induced $CD4^+CD25^+FoxP3^+$ Treg cells expansion (Fig. 3e).

SHH signaling-mediated expression of PD-L1 and COX-2-PGE_2 is required for Treg expansion. Having established that mycobacteria activate the PI3K-mTOR-NF-κB-SHH signaling cascade in human DCs to induce Treg expansion, we attempted to identify the molecular regulators that mediate the process. Utilizing cues from previous investigations[15–17], we analyzed the role for co-stimulatory molecules PD-L1, PD-L2 and a soluble factor such as COX-2-PGE_2 in the current study. Stimulation of DCs with *M. bovis* BCG and *M. tuberculosis*

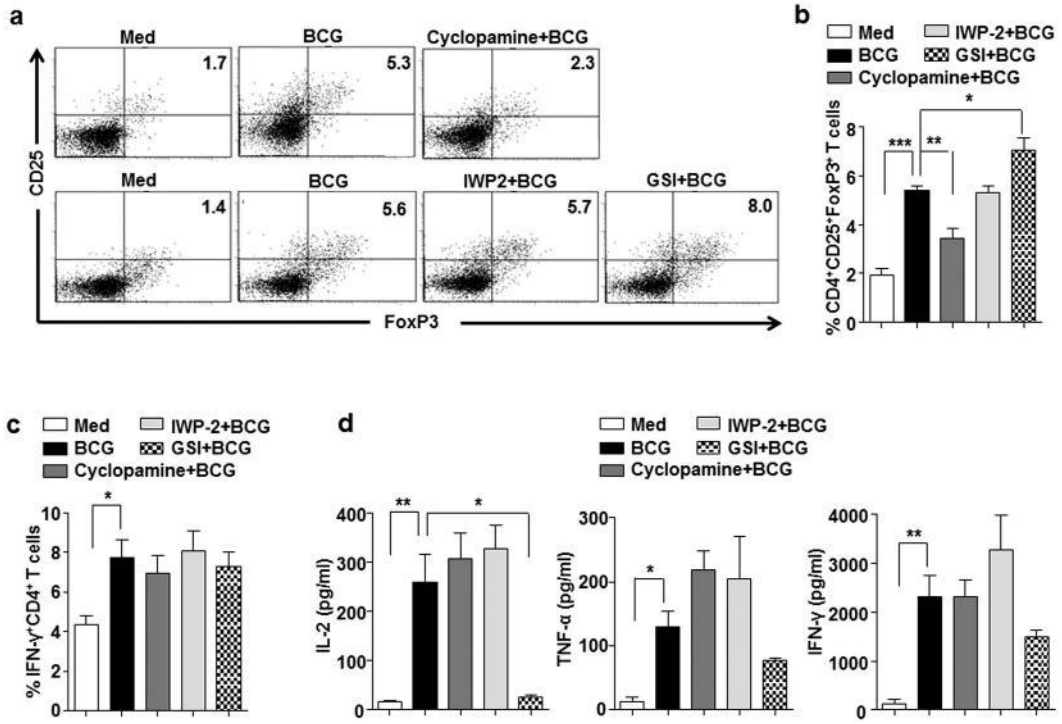

Figure 1. Opposing role of SHH and NOTCH1 signaling during *M. bovis* BCG-induced Treg expansion. (**a**,**b**) Immature DCs were cultured in GM-CSF and IL-4 alone (Med) or along with pharmacological inhibitors of SHH signaling pathway like Cyclopamine (SMO inhibitor), WNT signaling pathway like IWP-2 (a WNT secretion inhibitor) or NOTCH signaling like γ-secretase inhibitor (GSI) for 1 h followed by infection with *M. bovis* BCG (MOI 1:10) for 24 h. After extensive wash, DCs were co-cultured with autologous $CD4^+$ T cells. $CD4^+CD25^+FoxP3^+$ Tregs were analyzed by flow cytometry. (**a**) Representative dot blot of 6 independent experiments is shown. (**b**) Percentage of $CD4^+CD25^+FoxP3^+$ cells in the DC-$CD4^+$ T cell co-cultures (mean ± SEM, n = 8). (**c**) In a similar set up as panels (**a**,**b**) percentage of IFN-γ^+CD4^+ cells in the DC-$CD4^+$ T cell co-cultures (mean ± SEM, n = 7). (**d**) T cell cytokines IL-2, TNF-α and IFN-γ were analyzed in the cell-free culture supernatants of DC:T cell co-culture (mean ± SEM, n = 6–7) by cytokine bead array. Med, Medium. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (one-way ANOVA followed by Turkey's multiple-comparisons test).

resulted in increased expression of *PD-L1* and *PTGS2*/COX-2 (Fig. 4a, left panel). In line with our previous results, PD-L2 was not induced in stimulated DCs. Further, enhanced COX-2 expression was also associated with concomitant increase in PGE_2 secretion by DCs (Fig. 4a, right panel). Following the previous results, expression of PD-L1 and *PTGS2*/COX-2 transcripts was found to be PI3K-mTOR-NF-κB-SHH dependent (Fig. 4b). Further, surface expression of PD-L1 (Fig. 4c), expression of COX-2 in the whole cell lysate (Fig. 4d) and secretion of PGE_2 by DCs (Fig. 4e) followed the similar trend. SHH-mediated expression of these mediators was confirmed by reduced expression of PD-L1, COX-2 and PGE_2 on *M. bovis* BCG infection in the presence of *SHH*-specific siRNA (Fig. 4f–i). Finally, inhibition of either PD-L1 or COX-2 by specific blocking antibody (anti-PD-L1) or pharmacological inhibitor (NS-398, COX-2 inhibitor) could partially inhibit the *M. bovis* BCG-induced Treg expansion (Fig. 5a). Of note, inhibition of both PD-L1 and COX-2 in DCs significantly suppressed the ability of mycobacteria to expand Tregs (Fig. 5b,c).

GLI1, a zinc-finger protein, is a dedicated transcription factor for SHH signaling-mediated gene expression[31]. ChIP analysis revealed *M. bovis* BCG-induced recruitment of GLI1 to the promoter of *PTGS2*/COX-2 in DCs suggesting a SHH-dependent transcriptional regulation of COX-2 expression (Fig. 6a). However, no apparent change was observed on *PD-L1* promoter (Fig. 6a). In this context, we speculated the role for modulation of negative regulators of PD-L1 by infection-responsive SHH signaling. MiRNAs belong to one such class of post-transcriptional regulators. Extensive bioinformatic analysis (TargetScan, miRanda, miRWalk and RNAhybrid) together with available cues on downregulated miRNAs in tuberculosis patient[20,32] or *M. tuberculosis* or *M. bovis* BCG samples[33] identified miR-15b, miR-324-5p, miR-338-5p and miR-425-5p as candidate miRNAs that could target PD-L1. In line with this, DCs infected with *M. tuberculosis* or *M. bovis* BCG exhibited reduced levels of the identified miRNAs. MiR-155 was utilized as a known positive control (Fig. 6b). However, among the *M. bovis* BCG-downregulated miRNAs, expression of miR-324-5p and miR-338-5p was found to be SHH-regulated as the downregulation of miR-324-5p and miR-338-5p was significantly rescued in presence of a SHH inhibitor, cyclopamine (Fig. 6c). The target sites located at the residues spanning from 99–106 (for miR-324-5p) and 526–533 (for miR-338-5p) of the 3′UTR of *PD-L1* were identified as critical for miRNA-3′UTR interactions (Fig. 6d). To establish that *PD-L1* is the bonafide targets of miR-324-5p and miR-338-5p, we utilized the classical 3′UTR luciferase assays. Transfection of a monocytic cell line THP-1 with miR-324-5p or miR-338-5p

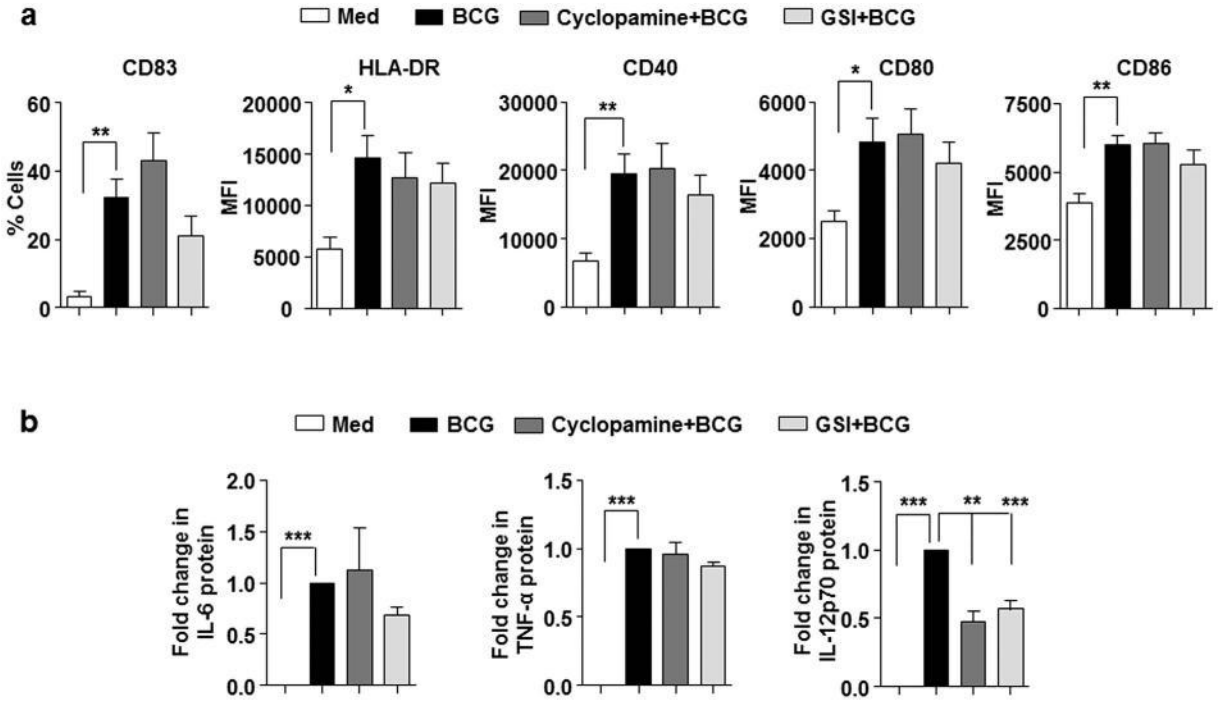

Figure 2. ***M. bovis*** **BCG-induced proinflammatory responses are moderately NOTCH dependent and suppressed by SHH.** DCs were cultured with GM-CSF and IL-4 and left untreated (Med) or infected with *M. bovis* BCG alone or after 1 h pretreatment with the indicated inhibitors for 24 h. (mean ± SEM, n = 6–10) (**a**) Surface expression of maturation markers CD83, HLA-DR, CD40, CD86 and CD80 were examined by flow cytometry. Data is represented as % positive cells or MFI. (**b**) Cell-free supernatants from the above-said experiment were assessed for secretion of IL-6, TNF-α, IL-12p70 by cytokine bead array (mean ± SEM, n = 5). Med, Medium. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (one-way ANOVA followed by Turkey's multiple-comparisons test).

mimics markedly reduced WT *PD-L1* 3′UTR luciferase activity. However, no significant reduction was observed when mutant construct for miR-324-5p and miR-338-5p binding on *PD-L1* 3′UTR was utilized (Fig. 6e). These results thus validate that *PD-L1* is a direct target of miR-324-5p and miR-338-5p. In accordance with this observation, we found that DCs expressing miR-324-5p or miR-338-5p miRNAs displayed reduced ability to induce the surface expression as well as total protein levels of PD-L1 on *M. bovis* BCG infection (Fig. 6f,g). Together, these results highlight a dichotomous role for *M. bovis* BCG-induced SHH signaling in DCs during expression of COX-2 and PD-L1.

NOTCH1 signaling-dependent PI3K-mTOR-NF-κB axis regulates the Th1 responses. After establishing the role for SHH signaling, the other identified signaling pathway (Fig. 1a,b) in DCs that affected the Treg expansion, NOTCH signaling, was analyzed. NOTCH1 signaling activation in DCs-infected with mycobacteria was assessed using transcript analysis of *HES1*, *NOTCH1*, *JAG1* and *JAG2* and generation of NICD. Elevated levels of *HES1* and *JAG2* transcripts and NICD marked the activation of NOTCH1 signaling on *M. tuberculosis* or *M. bovis* BCG infection (Fig. 7a,b). Exploring the role for other NOTCH receptor, it was found that while no increase in the transcripts of *NOTCH2-4* was observed on *M. bovis* BCG stimulation, intracellular domain of NOTCH2 and NOTCH4 were induced on mycobacterial infection (Fig. 7c,d). However, we chose to analyze the function of NOTCH1 signaling in the current study as its activation was found comparatively more robust. While inhibition of PI3K-mTOR-NF-κB axis did not alter the *M. bovis* BCG-induced NICD generation (Fig. 7e), pharmacological intervention of NOTCH1 signaling using GSI significantly abrogated the *M. bovis* BCG-mediated activation of PI3K-mTOR-NF-κB pathway suggesting that NOTCH1 pathway regulates the PI3K-mTOR-NF-κB signaling cascade (Fig. 7f). In line with this observation, NOTCH1-responsive PI3K-mTOR-NF-κB axis was found essential for generation of *M. bovis* BCG-induced inflammatory cytokines like IL-6, TNF-α and IL-12 despite for the fact that no change in the infection-induced DC maturation was observed when PI3K-mTOR-NF-κB pathway was inhibited (Fig. 7g).

Counter-regulation of SHH and NOTCH1 signaling during *M. bovis* BCG infection. Previously, we found that NOTCH1 signaling negatively regulated the *M. bovis* BCG-mediated $CD4^{+}CD25^{+}FoxP3^{+}$ Treg expansion (Fig. 1a,b). In accordance with this observation, DCs treated with GSI exhibited increased expression of PD-L1 on *M. bovis* BCG infection (Fig. 8a,b). This suggests that *M. bovis* BCG-induced NOTCH1 signaling negatively regulates PD-L1 expression to regulate Treg expansion. On the other hand, DCs treated with cyclopamine displayed enhanced expression of *JAG2* transcript (Fig. 8c) and increased NICD formation (Fig. 8d,e).

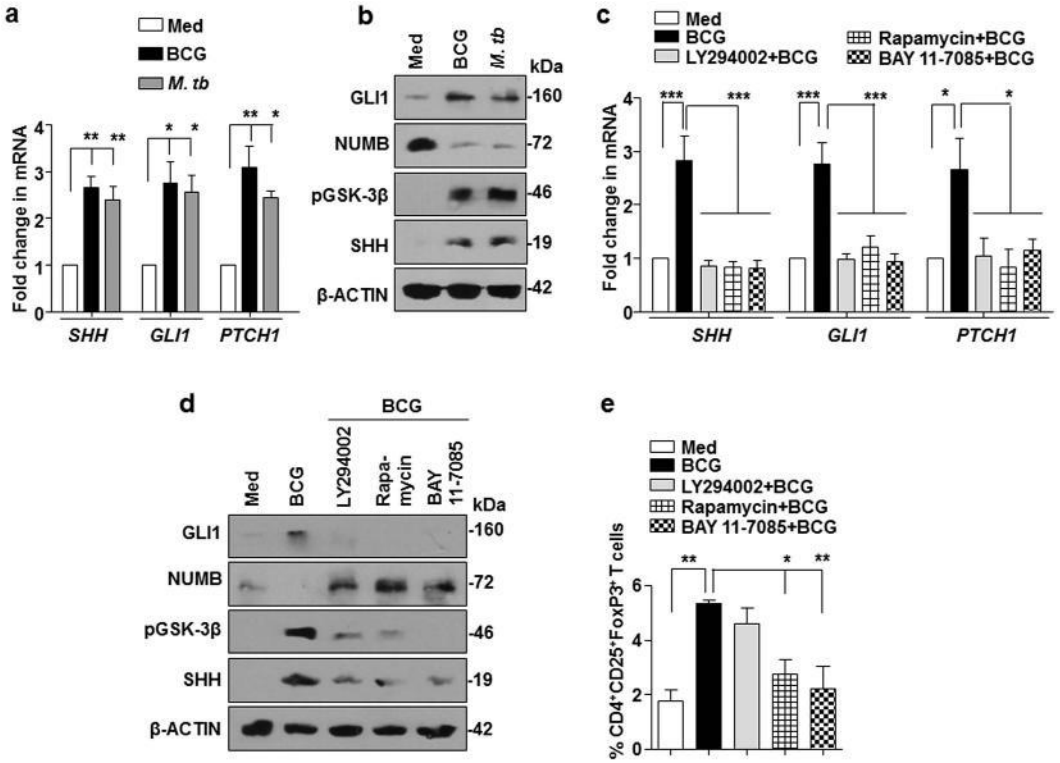

Figure 3. PI3K-mTOR-NF-κB pathway mediates *M. bovis* BCG-induced SHH signaling and Treg expansion. (**a,b**) Five-day-old differentiated immature DCs were infected with 1:10 MOI of *M. bovis* BCG or *M. tuberculosis* H37Ra for 6 h. Transcript (**a**) and protein (**b**) levels of various SHH signaling markers were determined using quantitative real time RT-PCR and immunoblotting respectively. (**c,d**) Expression analysis of SHH signaling markers in immature DCs pretreated with the indicated pharmacological inhibitors for 1 h prior to 6 h infection with *M. bovis* BCG. (**e**) DCs infected with *M. bovis* BCG alone or after pretreatment of LY294002 (PI3K inhibitor), Rapamycin (mTOR inhibitor) or BAY 11-7085 (NF-κB inhibitor) were co-cultured with autologous $CD4^+$ T cells. Percentage of $CD4^+CD25^+FoxP3^+$ T cells (mean ± SEM, n = 4) were analyzed by flow cytometry. All RT-PCR data represents the mean ± SEM from at least 3 independent experiments and all blots are representative of 3 independent experiments. Images have been cropped for presentation; full-size blot is shown in Supplementary Fig. S1. Med, Medium. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (one-way ANOVA followed by Turkey's multiple-comparisons test).

Together, these results indicate a counter-balance between the effects of infection-induced NOTCH1 and SHH signaling cascades to regulate $CD4^+CD25^+FoxP3^+$ Treg expansion.

Discussion

Induction and expansion of an inhibitory $CD25^+FoxP3^+$ Treg population reckons as one of the immune evasion strategies that mycobacteria employ to combat the protective immune responses[5,9,15,16]. In the current investigation, we found novels roles for SHH and NOTCH1 pathways in modulating *M. bovis* BCG-induced Treg expansion. We attribute the current observation to Treg expansion and not Treg induction as co-culture experiments with naïve $CD4^+$ T did not show significant change in Treg population on mycobacterial infection (data not shown).While no previous reports were available implicating SHH signaling regulating Treg functions, multiple studies in mice and humans have identified NOTCH signaling to modulate Treg population. Overexpression of JAG1 signaling in DCs induced a regulatory phenotype in $CD4^+$ T cells in both humans and mice[34,35]. However, direct role of JAG1 assisting the differentiation of naive $CD4^+$ T to Tregs is not established, underscoring the differential functions of JAG1 in regulating Treg generation in humans and mice[36]. In another investigation, NOTCH1 signaling was found to promote TGF-β-mediated Treg functions in both humans and mice[26]. On the contrary, DLL4-mediated NOTCH signaling inhibits TGF-β-mediated Treg development and JNK-responsive STAT5 activation, a requisite for FoxP3 expression and maintenance[25,37]. In line with latter observation, mycobacteria-responsive activation of NOTCH1-JAG2 axis in DCs was found to suppress $CD25^+FoxP3^+$ Treg population. Supporting our observation, while JAG2 was found to expand Tregs during graft rejection in mice[38], elevated JAG2 levels in hematopoietic progenitor cells was required for Treg expansion in mice to suppress T cell-mediated diseases[39]. Significant contribution of the WNT pathway in Treg expansion was not observed. However, previous reports suggest both positive and negative regulation of Treg functions by the canonical WNT signaling pathway. While inhibition of β-CATENIN in DCs comprised their ability to induce Tregs[27], WNT signaling activation inhibited the transcriptional activity of FoxP3 in T cells[28].

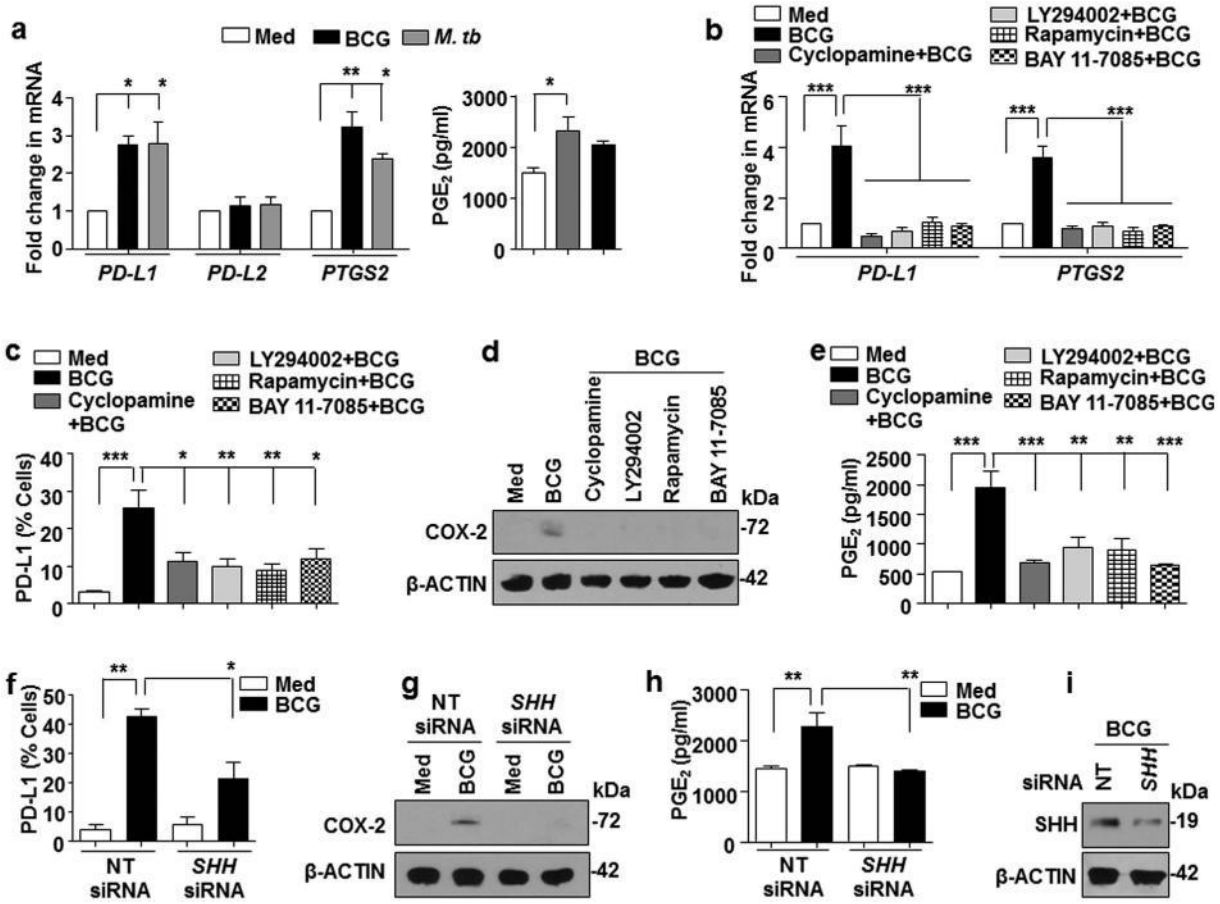

Figure 4. ***M. bovis*** **BCG-induced PD-L1 and COX-2 are SHH signaling dependent.** (**a,b**) Quantitative real time RT-PCR for expression analysis of *PD-L1*, *PD-L2*, *PTGS2*/COX-2 and ELISA from cell-free supernatants to estimate the secretion of PGE_2 on (**a**) infection of immature DCs with *M. bovis* BCG or *M. tuberculosis* H37Ra for 12 h or (**b**) infection of indicated pharmacological inhibitor treated immature DCs with *M. bovis* BCG for 12 h. (**c**) DCs were cultured in GM-CSF and IL-4 alone (Med) or with pharmacological inhibitor followed by *M. bovis* BCG as indicated for 24 h. Surface expression of PD-L1 as % positive cells was analyzed by flow cytometry (mean ± SEM, n = 8). (**d,e**) DCs were treated as explained above. Immunoblotting for COX-2 from total cell lysate (**d**) and ELISA for measuring PGE_2 in the cell-free supernatant (**e**). (**f–i**) Immature human DCs were transiently transfected with NT or *SHH* siRNA. 48 h post transfection, cells were infected with *M. bovis* BCG for 24 h to assess the surface expression of PD-L1 by flow cytometry (mean ± SEM, n = 4) (f) or 12 h to estimate COX-2 protein by immunoblotting (**g**) and PGE_2 in the cell-free supernatant by ELISA (**h**). Validation of *SHH* siRNA was performed by immunoblotting for SHH in the siRNA-transfected DCs (**i**). All RT-PCR and ELISA data represents the mean ± SEM from at least 3 independent experiments and all blots are representative of 3 independent experiments. Images have been cropped for presentation; full-size blot is shown in Supplementary Fig. S1. Med, Medium; NT, Non-targeting. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (one-way ANOVA followed by Turkey's multiple-comparisons test).

M. bovis BCG-induced NOTCH1-PI3K-mTOR-NF-κB signaling in DCs was also found to promote inflammatory cytokines like IL-1β, IL-6, TNF-α and IL-12. Multiple investigations in both humans and mice have subscribed a NOTCH signaling ligand-specific T cell differentiation[40]: Th1[41–43], Th2[44–46] and Th17[47,48]. However, in the current investigation, mycobacterial infection of human DCs induced NOTCH1 signaling to program the cells towards pro-inflammatory in nature. This observation is in accordance with our previous report that suggested a function of a mycobacterial immunodominant protein, Rv0754, in regulating a NOTCH1-PI3K pathway-dependent pro-inflammatory environment to subvert CTLA-4- and TGF-β-induced suppression of DC maturation[22]. Interestingly, though infection-induced signaling pathways regulated the DC functions in terms of modulating the specific cytokines and T cell phenotype, no apparent role for these identified pathways was found for DC maturation.

To identify the molecular mechanism that mediates SHH and NOTCH signaling-responsive T cell phenotype during mycobacterial infection, we chose to analyze the contributions of PD-L1, PD-L2 and PGE_2. The co-stimulatory molecules on the APCs, PD-L1 and PD-L2 signal to the PD-1 on the T cells to orchestrate a Treg differentiation and expansion[49]. Formation of the PD-L1 and PD-1 ligand-receptor complex triggers the SHP1/2 activation that suppresses the STAT1 activity, thereby abrogating IFN-γ-mediated responses. STAT1 is

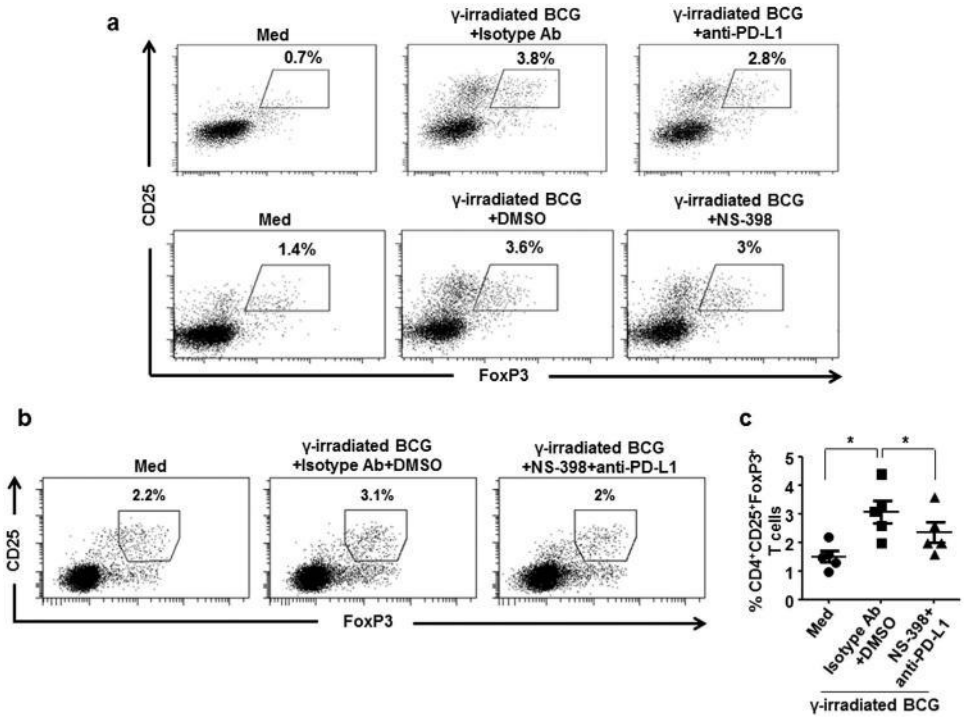

Figure 5. PD-L1 and COX-2 mediate *M. bovis* BCG-induced Treg expansion. (**a**) Inhibition of either PD-L1 or COX-2, partially inhibit the BCG-induced Treg expansion. γ-irradiated BCG-stimulated DCs were incubated with anti-PD-L1 blocking antibody or isotype antibody (upper panel). Alternatively, DCs were pretreated with DMSO or NS-398 (COX-2 inhibitor) before stimulation with BCG (lower panel). After extensive wash, DCs were co-cultured with autologous $CD4^+$ T cells for five days. Representative dot-plots showing frequency of $CD4^+CD25^+FoxP3^+$ Tregs were presented. The Treg response induced by DCs cultured in medium alone is represented by 'Med'. (**b,c**) Inhibition of both PD-L1 and COX-2 in DCs significantly suppress the ability of BCG to expand Tregs. DCs were pretreated with DMSO or NS-398 before stimulation with BCG. After extensive washing, these DCs were incubated with anti-PD-L1 blocking antibody or isotype antibody and co-cultured with autologous $CD4^+$ T cells for five days. $CD4^+CD25^+FoxP3^+$ Tregs were analyzed by flow cytometry. Representative dot-plots showing frequency of $CD4^+CD25^+FoxP3^+$ Tregs were presented (**b**). (**c**) Percentage of $CD4^+CD25^+FoxP3^+$ cells in the DC-$CD4^+$ T cell co-cultures (mean ± SEM, n = 5). $^*P < 0.05$ (one-way ANOVA followed by Holm-Sidak's multiple comparisons test).

otherwise known to inhibit FoxP3 expression[50]. Importantly, reports suggest that induced expression of PD-L1 in human DCs is necessary for mycobacteria-induced Treg expansion[15,16]. However, PD-L1 KO and PD-1 KO mice strangely displayed exacerbated tuberculosis disease with excessive inflammatory responses and increased susceptibility to infection underscoring the crucial role for PD-L1 signaling during mycobacterial infection[14,51]. Interestingly, inhibition of PD-L1 in DCs also promoted mycobacteria-induced IFN-γ production in T cells[52]. Of note, COX-2 catalyzed PGE_2 serves as a cue for Treg expansion and functions[53,54]. PGE_2-EP signaling is known to aid in FoxP3 expression[55]. Importantly, in humans, mycobacterial infection triggers PGE_2-dependent expansion of $CD25^+FoxP3^+$ Tregs[17]. In agreement with all these observations, our results revealed that SHH-dependent expression of PD-L1 and COX-2-PGE_2 during mycobacterial infection induces the Treg expansion. It was also noted that there was synergistic effect of PD-L1 and COX-2 in mediating mycobacteria-induced Treg expansion. In fact, upon inhibition of both PD-L1 and COX-2, the Treg frequency reached close to medium control conditions. Interestingly, a recent report showed similar synergistic effects of COX and PD-1 for eradication of tumors and its usefulness as adjuvants for immune-based therapies[56]. Further, while expression of COX-2 was a transcriptional regulation by SHH signaling, post-transcriptional regulation of PD-L1 by SHH signaling-regulated miRNAs was observed.

Based on available information on genome-wide miRNA profiling in tuberculosis patients vs healthy individuals[20,32,57,58] and *ex-vivo* infection studies[33], a panel of miRNAs that were downregulated on mycobacterial infection and served as putative miRNAs that target PD-L1 were chosen for the study. MiR-155 was utilized as a positive control as it is not only known to be induced to several manifolds during mycobacterial infection, but also associated with maturation of DCs[59,60]. Among the tested miRNAs, miR-324-5p and miR-338-5p were identified as the bonafide miRNAs that target PD-L1 in a SHH-dependent manner and hence, downregulated during mycobacterial infection. However, the mechanism of SHH-mediated transcriptional regulation of miR-324-5p and miR-338-5p needs to be studied further.

Mycobacteria harbors numerous antigens that are recognized by several pattern recognition receptors; TLR2 being the dominant one. Various studies, including ours, have suggested that mycobacteria and its antigens

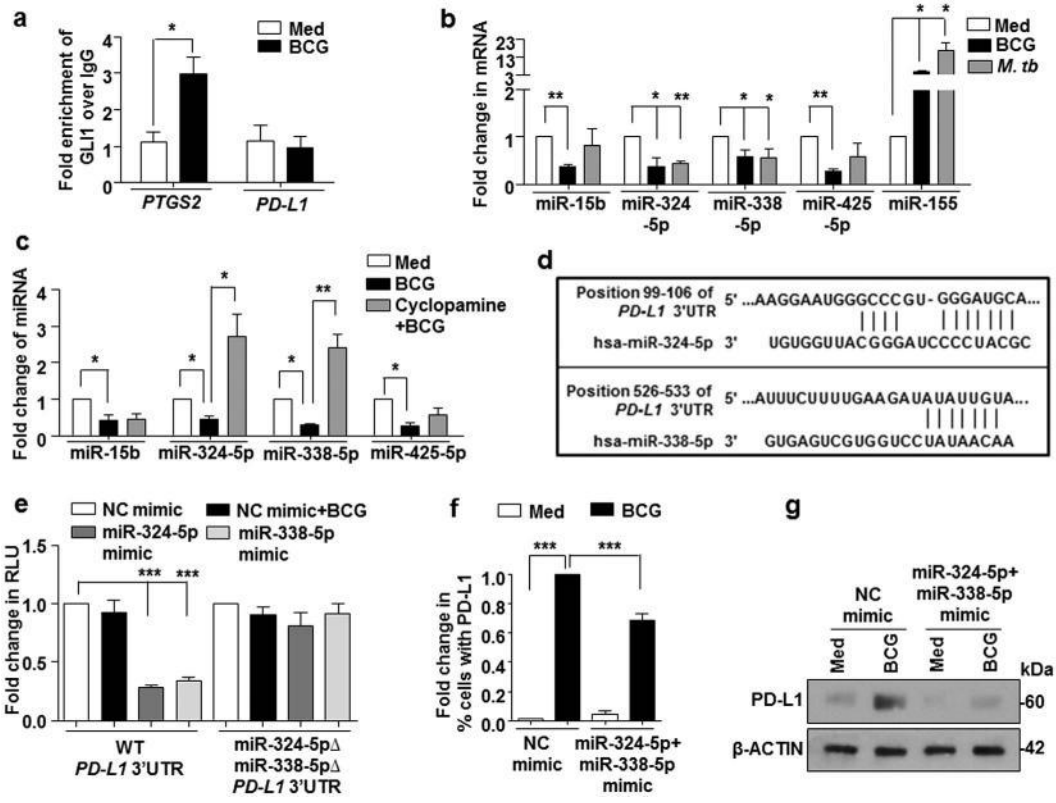

Figure 6. Bi-functional role of SHH signaling to regulate PD-L1 and COX-2 expression. (**a**) The recruitment of GLI1 at human *PTGS2* and *PD-L1* promoter upon infection with *M. bovis* BCG for 12 h in immature DCs was evaluated by ChIP assay. (**b,c**) DCs were infected with *M. bovis* BCG or *M. tuberculosis* H37Ra alone (**b**) or with pharmacological inhibitors of SHH pathway (**c**) and quantitative real time RT-PCR analysis was performed on total RNA isolated using indicated miRNA-specific primers. (**d**) Putative miR-324-5p and miR-338-5p binding sites in the 3′UTR of *PD-L1*. (**e**) THP-1 cells were transfected with WT *PD-L1* 3′UTR or miR-324-5pΔ miR-338-5pΔ *PD-L1* 3′UTR constructs with miR-324-5p or miR-338-5p mimics as indicated. Transfected THP-1 cells were further stimulated with *M. bovis* BCG as indicated and luciferase assay was performed. (**f,g**) DCs transfected with control or miR-324-5p and miR-338-5p mimics were infected with *M. bovis* BCG for 24 h (**f**) or 18 h (**g**) as indicated. While surface expression of PD-L1 was evaluated by flow cytometry (mean ± SEM, n = 3) (**f**), protein levels of PD-L1 in the total cell lysate was assessed by immunoblotting (**g**). All RT-PCR and luciferase data represents the mean ± SEM from 3 independent experiments. Images have been cropped for presentation; full-size blot is shown in Supplementary Fig. S1. Med, Medium; NC, Negative control. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (Student's *t*-test for Panel a, one-way ANOVA followed by Turkey's multiple-comparisons test for others).

induce TLR2 signaling in both macrophages and DCs[16,18,19,21–23,57,61]. Importantly, many of these previous studies have implicated that mycobacteria-induced activation of NOTCH in both macrophages[18,62] and DCs[22] and mycobacteria-induced activation of SHH pathways in macrophages[20,21] were TLR2-dependent. Further, while current and previous results[18,22] suggest that mycobacteria-induced NOTCH1 signals the activation of PI3K-mTOR-NF-κB axis, current and a previous study[20] also suggests that mycobacteria-induced SHH signaling is PI3K-mTOR-NF-κB dependent. Keeping these results in mind, TLR2-dependent activation of NOTCH1 signaling during mycobacterial infection could play a dominant role in DCs. However, we have not assessed the role of TLR2 in the current study.

In summary, establishing novel roles for SHH and NOTCH1 signaling pathways, we found mycobacteria-activated SHH signaling induces PD-L1 and COX-2-PGE_2 to mediate the expansion of $CD4^+CD25^+FoxP3^+$ Tregs whereas infection-induced NOTCH1 signaling was found to suppress the Treg expansion (Fig. 8f). Thus, mycobacteria modulate the host signaling pathways and molecular regulators in DCs to determine the functional outcome of the immune responses including Tregs expansion.

Methods

Antibodies. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) to CD86, CD1a, PD-L1 (CD274), IFN-γ (all anti-human), phycoerythrin (PE)-conjugated mAbs to CD80, CD83 and CD25, APC-conjugated HLA-DR (all anti-human), anti-human Alexa Fluor 700-CD4 were from BD Biosciences, and PE-conjugated mAb to anti-human CD40 was from Becton Dickinson. Human Foxp3-APC, anti-PD-L1 and isotype control were from eBioscience. CD14 magnetic beads, T cell isolation kit II, GM-CSF and IL-4 were from

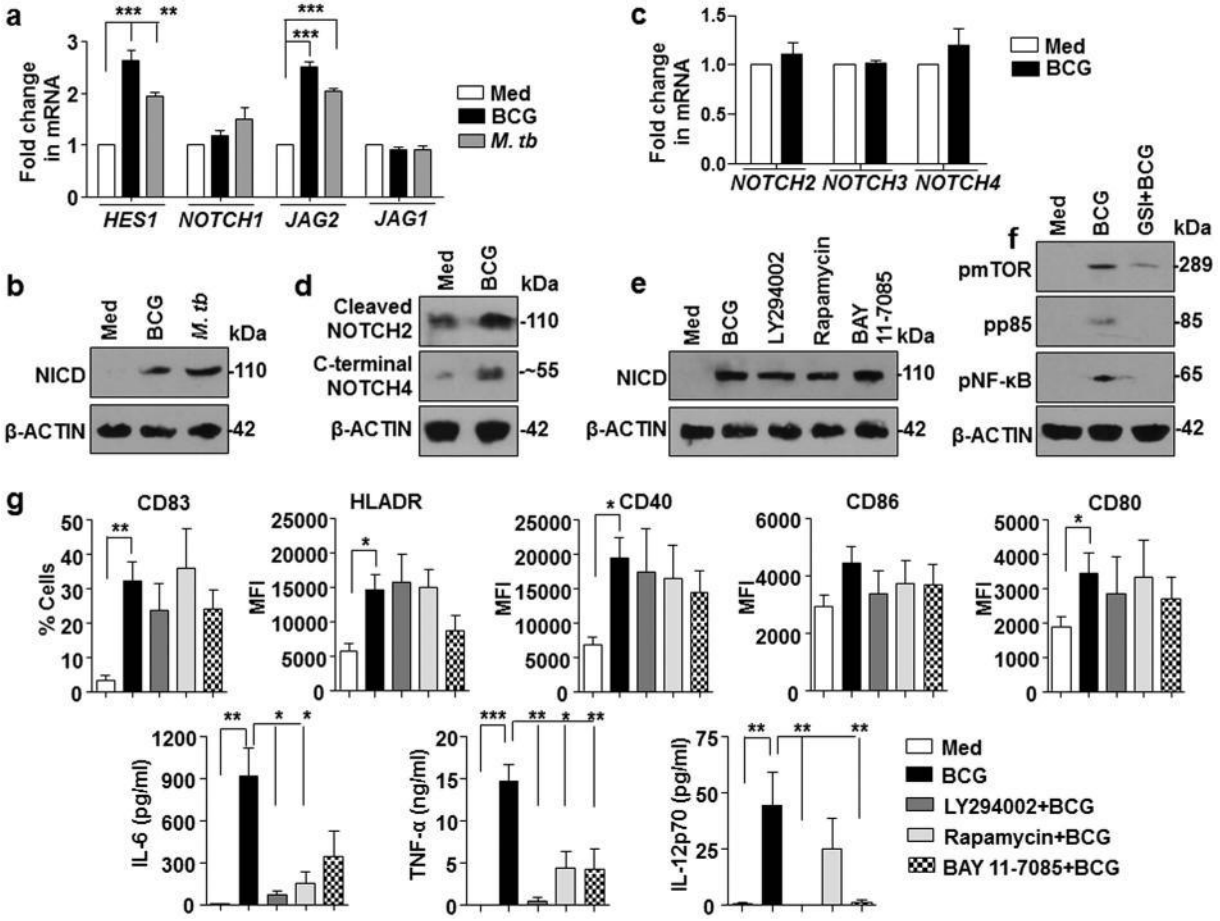

Figure 7. ***M. bovis*** **BCG-induced NOTCH1 signaling in DCs regulates PI3K-mTOR-NF-κB pathway.** (**a**) Quantitative real time RT-PCR for assessing NOTCH1 signaling markers, *HES1*, *NOTCH1*, *JAG1* and *JAG2* on infection of immature DCs with *M. bovis* BCG or *M. tuberculosis* H37Ra for 6 h. (**b**) Activation of NOTCH1 signaling was determined by immunoblotting for NICD in DCs infected with *M. bovis* BCG or *M. tuberculosis* H37Ra. (**c,d**) Transcript (**c**) and intracellular domain (**d**) of NOTCH2-4 on *M. bovis* BCG stimulation was analyzed by quantitative real time RT-PCR (**c**) or immunoblotting (**d**). (**e**) NOTCH1 signaling activation was assessed by immunoblotting for NICD in DCs pretreated with the indicated pharmacological inhibitors and infected with *M. bovis* BCG. (**f**) Immunoblotting for evaluating PI3K-mTOR-NF-κB pathway activation using DCs infected with *M. bovis* BCG with or without GSI (NOTCH signaling inhibitor). (**g**) DCs were cultured with GM-CSF and IL-4 and left untreated (Med) or infected with *M. bovis* BCG alone or after 1 h pretreatment with the indicated inhibitors for 24 h. Surface expression of maturation markers CD83, HLA-DR, CD40, CD86 and CD80 were examined by flow cytometry (mean ± SEM, n = 7–10). Data is represented as % positive cells or MFI ((**g**), top panels). Cell-free supernatants from the above-said experiment were assessed for secretion of IL-6, TNF-α, IL-12p70 by cytokine bead array ((**g**), lower panels) (mean ± SEM, n = 5–7). All RT-PCR data represents the mean ± SEM from at least 3 independent experiments and all blots are representative of 3 independent experiments. Images have been cropped for presentation; full-size blot is shown in Supplementary Fig. S2. Med, Medium. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (one-way ANOVA followed by Turkey's multiple-comparisons test).

Miltenyi Biotec. Anti-β-ACTIN and anti-PGE_2 antibodies were purchased from Sigma-Aldrich. Anti-SHH, anti-GLI1, anti-NUMB, anti-Ser9 phospho-GSK-3β, anti-NICD (Cleaved Notch1), anti-Ser2448 phospho mTOR, anti-Tyr458 phospho p85 and anti-Ser536 phospho NF-κB p65 were purchased from Cell Signaling Technology. Anti-COX-2 was from Calbiochem. Anti-Notch 2 intracellular domain and anti-Notch 4 -C-terminal antibodies were from Abcam. HRP conjugated anti-rabbit IgG and anti-mouse IgG was obtained from Jackson ImmunoResearch.

Generation of Human DCs. Human Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of the healthy blood donors purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (N°12/EFS/079). All samples were analyzed anonymously. Circulating monocytes were isolated from these PBMCs using CD14 magnetic beads. The purity was more than 98%. Monocytes were cultured in RMPI-1640 medium containing

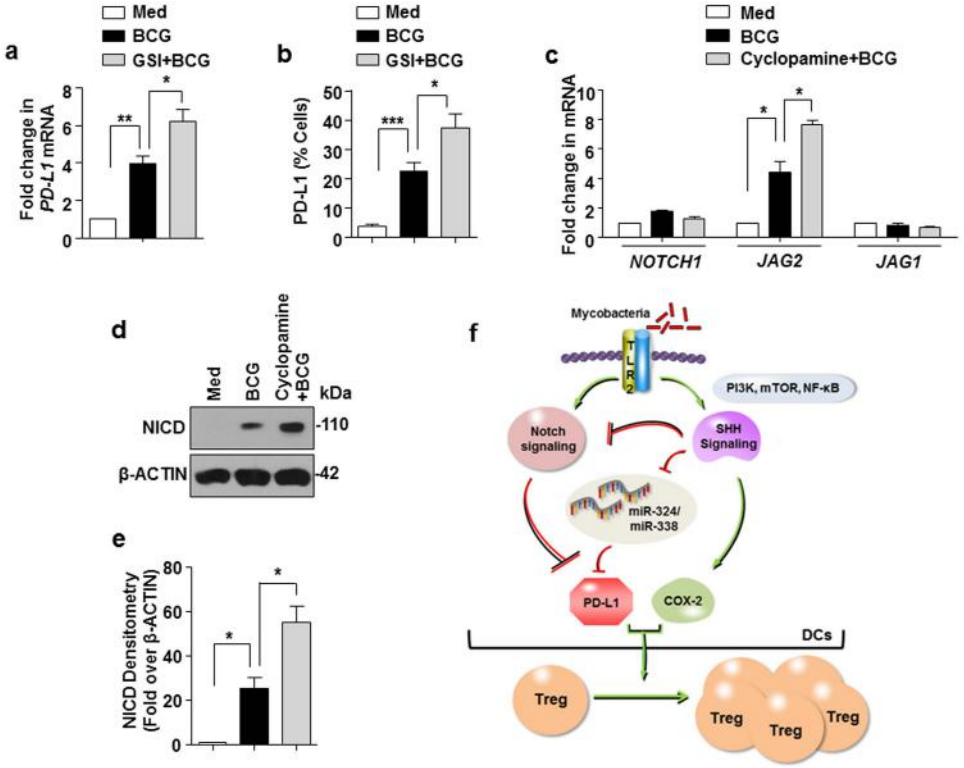

Figure 8. Counter-regulation of NOTCH1 and SHH pathway functions during *M. bovis* BCG infection of DCs. (**a**,**b**) Transcript (**a**) and surface (**b**) expression of PD-L1 in DCs infected with *M. bovis* BCG alone or after 1 h pretreatment of GSI by quantitative real time RT-PCR and flow cytometry (mean ± SEM, n = 7) respectively. (**c**–**e**) Immature DCs were pretreated with SHH signaling inhibitors and infected with *M. bovis* BCG for 6 h. NOTCH1 signaling markers, *NOTCH1*, *JAG1* and *JAG2* transcripts were analyzed using quantitative real time RT-PCR (**c**), NICD by immunoblotting (**d**) and densitometric analysis of panel D (**e**). (**f**) Model: schematic representation of the obtained results. All RT-PCR and densitometry data represents the mean ± SEM from at least 3 independent experiments and all blots are representative of 3 independent experiments. Images have been cropped for presentation; full-size blot is shown in Supplementary Fig. S2. Med, Medium. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$ (one-way ANOVA followed by Turkey's multiple-comparisons test).

10% FCS in the presence of GM-CSF (1000 IU/10^6 cells) and IL-4 (500 IU/10^6 cells) for 5 days to obtain immature DCs and used for subsequent experiments.

Stimulation of DCs with mycobacteria. 0.5×10^6 immature DCs (FACS, Co-culture experiments) or 10^6 immature DCs (Immunoblotting, RNA isolation, transfections experiments) or 2×10^6 immature DCs (ChIP experiments) were washed and infected with 1:10 multiplicity of infection (MOI) of *M. bovis* BCG or *M. tuberculosis* H37Ra for indicated time in the presence of GM-CSF and IL-4. Supernatants were collected for analyzing cytokines and cells were utilized for further indicated assays. *M. bovis* BCG Pasteur 1173P2 was obtained from Pasteur Institute, Paris, France and *M. tuberculosis* H37Ra was kind research gift from Dr. P. Ajitkumar, IISc, India. Mycobacteria were grown in Middlebrook 7H9 broth to mid-log phase and then aliquoted, following which it was stored at −70 °C. Representative vials were thawed and the mycobacterial cells' viability was then assessed by plating on Middlebrook 7H10 agar plates.

Treatment with pharmacological reagents. Cells were treated with the given inhibitor (from Calbiochem) for 1 h before experimental treatments at following concentrations: Cyclopamine (15 μM); IWP-2 (5 μM); GSI (750 nM); LY294002 (10 μM); Rapamycin (200 nM); BAY 11-7085 (5 μM); NS-398 (25 μM). DMSO at 0.1% concentration was used as the vehicle control. In all experiments involving pharmacological reagents, a tested concentration was used after careful titration experiments assessing the viability of the DCs using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Transient transfection studies. Transient transfection of immature DCs with 100 nM siRNA or 150 nM miRNA mimics were carried out utilizing Oligofectamine reagent (Life Technologies). *SHH*, non-targeting siRNA and siGLO Lamin A/C were obtained from Dharmacon as siGENOME™ SMARTpool reagents, which contain a pool of four different double-stranded RNA oligonucleotides. MiR-324-5p, miR-338-5p mimics and negative control mimics were purchased from Ambion (Life Technologies). Transfection efficiency was found to be more than 50% in all the experiments as determined by counting the number of siGLO Lamin A/C positive cells in a

microscopic field using fluorescent microscope. 48 h post siRNAs transfection or 24 h post miRNAs transfection, the cells were treated or infected as indicated and processed for analysis.

RNA isolation and quantitative real time RT-PCR. DCs were treated or infected as indicated and total RNA from the cells was isolated by TRI reagent (Sigma-Aldrich). 2 μg of total RNA was converted into cDNA using First strand cDNA synthesis kit (Invitrogen). Quantitative real time RT-PCR was performed using SYBR Green PCR mixture (KAPA Biosystems) for quantification of the target gene expression. All the experiments were repeated at least three times independently to ensure the reproducibility of the results. *GAPDH* was used as internal control. The primers used for PCR amplification are: *GAPDH* forward 5′-ggagcgagatccctccaaaat-3′, *GAPDH* reverse 5′-ggctgttgtcatacttctcatgg-3′; *PD-L1* forward 5′-ggacaagcagtgaccatcaag-3′, *PD-L1* reverse 5′-cccagaattaccaagtgagtcct-3′; *PD-L2* forward 5′-accgtgaaagagccactttg-3′, *PD-L2* reverse 5′-gcgaccccatagatgattatgc-3′; *PTGS2* forward 5′-ggtggagaagtgggttttca-3′, *PTGS2* reverse 5′-gactcctttctccgcaacag-3′; *SHH* forward 5′-ctcgctgctggtatgctcg-3′, *SHH* reverse 5′-atcgctcggagtttctggaga-3′; *GLI1* forward 5′-agcgtgagcctgaatctgtg-3′, *GLI1* reverse 5′-cagcatgtactgggctttgaa-3′; *PTCH1* forward 5′-ccagaaagtatatgcactggca-3′; *PTCH1* reverse 5′-gtgctcgtacatttgcttggg-3′; *HES1* forward 5′-acacgacaccggataaaccaa-3′, *HES1* reverse 5′-gccgccagctatctttcttca-3′; *NOTCH1* forward 5′-gaggcgtggcagactatgc-3′, *NOTCH1* reverse 5′-cttgtactccgtcagcgtga-3′; *JAG1* forward 5′-tcgggtcagttcgagttgga-3′, *JAG1* reverse 5′-aggcacactttgaagtatgtgtc-3′; *JAG2* forward 5′-tgggactgggacaacgatac-3′, *JAG2* reverse 5′-agtggcgctgtagtagttctc-3′; *NOTCH2* forward 5′-tattgatgactgccctaaccaca-3′, *NOTCH2* reverse 5′-atagcctccattgcggttgg-3′; *NOTCH3* forward 5′-aagtcggggcacagtttctc-3′, *NOTCH3* reverse 5′-ctcccactcaccgatctgg-3′; *NOTCH4* forward 5′-gatgggctggacacctacac-3′, *NOTCH4* reverse 5′-cacacgcagtgaaagctacca-3′.

Quantification of miRNA expression. For detection of miRNAs, total RNA was isolated from infected or treated DCs using the TRI reagent. Quantitative real time RT-PCR for miR-155, miR-15b, miR-324-5p, miR-338-5p and miR-425-5p was performed using TaqMan miRNA assays (Ambion) as per manufacturer's instructions. U6 snRNA was used for normalization.

Immunoblotting. Infected or treated DCs were lysed in RIPA buffer constituting 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml of each aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4 and 1 mM NaF. Equal amount of protein from each cell lysate was resolved on a 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (PVDF) (Millipore) by the semi-dry transfer (Bio-Rad) method. Nonspecific binding was blocked with 5% nonfat dry milk powder in TBST [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.1% Tween 20] for 60 min. The blots were incubated overnight at 4 °C with primary antibody followed by incubation with anti-rabbit-HRP or anti-mouse-HRP secondary antibody in 5% BSA for 2 h. After washing in TBST, the immunoblots were developed with enhanced chemiluminescence detection system (Perkin Elmer) as per manufacturer's instructions. β-ACTIN was used as loading control. For probing another protein in the same region of the PVDF membrane, the blots were stripped in the stripping buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS and 0.7% β-mercaptoethanol] at 60 °C on a shaker, blocked with 5% nonfat dry milk powder and probed with antibodies as mentioned above.

DC:$CD4^+$ T cell co-cultures. Autologous $CD4^+$ T cells were obtained using T cell isolation kit II. The treated DCs were co-cultured with $CD4^+$ T cells at 1:10 for 5 days. Cell-free culture supernatants were collected for the analysis of T cell cytokines. Cells were treated with PMA-Ionomycin and Golgistop for 5 h. Following this, the T cells were processed for staining with fluorchrome-conjugated mAbs for flow cytometry.

γ-irradiated BCG-stimulated DC:$CD4^+$ T cell co-culture. Immature DCs treated with DMSO or 25 μM NS-398 for 1 h were stimulated with 20 μg/ml γ-irradiated BCG (Strain AF 2122/97 (ATCC® BAA-935™) obtained through BEI Resources, NIAID, NIH) for 24 h. Post stimulation, DCs were thoroughly washed with RPMI, counted and blocked with isotype control or anti-PD-L1 (10 μg/ml) for 1 h. DCs were then co-cultured with autologous $CD4^+$ T cells at 1:10 ratio for 5 days. On day 0 and day 3 of the co-culture, 25 μM NS-398 was added for efficient COX-2 inhibition. T cells were processed for staining with fluorchrome-conjugated mAbs for flow cytometry.

Flow cytometry. Surface staining of CD1a, HLA-DR, CD40, CD83, CD80 and CD86 on DCs was performed using fluorochrome-conjugated mAbs. T cells were surface-labeled with CD25- and CD4-specific fluorochrome-conjugated mAbs and then washed, fixed, permeabilized for intracellular staining of FoxP3. Cells were further processed for flow cytometry wherein 5000 gated events were recorded for each sample acquired in BD LSR II or BD FACSCanto II and the data was analyzed using FACSDiva™ software (BD Biosciences).

Cytokine analysis. Cytokines were quantified in the cell-free culture supernatants using BD™ Cytometric Bead Array (CBA) human inflammatory cytokine kits (IL-1β, CXCL8/IL-8, TNF-α, IL-6, IL-10 and IL-12p70) and human Th1/Th2 cytokine kits (IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ) (BD Biosciences). The data were analyzed using FCAP Array™ software (BD Biosciences).

Enzyme immunoassay for PGE_2. Enzyme immunoassays for quantitation of PGE_2 were carried out in 96-well microtiter plates (Nunc) using culture supernatant. ELISA plates were incubated with culture supernatant overnight at 4 °C followed by three washes with 1X PBST. After blocking with 1% BSA for 1 h at 37 °C, wells were incubated with anti-PGE_2 antibod y for 6 h at 37 °C followed by washing with 1X PBST. The plates were further incubated with HRP conjugated anti-rabbit secondary antibody for 2 h at 37 °C. The assay was developed with 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich). The absorbance values were measured at 450 nm by using ELISA reader (Molecular Devices).

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were carried out using a protocol provided by Upstate Biotechnology and Sigma-Adrich with certain modifications. Briefly, DCs were fixed with 1.42% formaldehyde for 15 min at room temperature followed by inactivation of formaldehyde with addition of 125 mM glycine. Nuclei were lysed in 0.1% SDS lysis buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM HEPES (pH 6.5), 0.1% SDS, 10 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 μg/ml of each aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4 and 1 mM NaF]. Chromatin was sheared using Bioruptor Plus (Diagenode) at high power for 40 rounds of 30 sec pulse ON/45 sec OFF. Chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using specific antibodies or rabbit preimmune sera according to the experiment complexed with Protein A agarose beads (Bangalore Genei). Immunoprecipitated complexes were sequentially washed [Wash Buffer A: 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS and protease/phosphatase inhibitors; Wash Buffer B: 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% Sodium deoxycholate and protease/phosphatase inhibitors; TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and eluted in elution buffer [1% SDS, 0.1 M $NaHCO_3$]. After treating the eluted samples with RNase A and Proteinase K, DNA was precipitated using phenol-chloroform-ethanol method. Purified DNA was analyzed by quantitative real time RT-PCR. All values in the test samples were normalized to amplification of the specific gene in Input and IgG pull down and represented as fold enrichment. All ChIP experiments were repeated at least three times and the primers utilized for GLI1 binding at *PTGS2* promoter are forward 5′-aaattgcgtaagcccggtggg-3′, reverse 5′-gacatctggcggaaacctgtgc-3′ and at *PD-L1* promoter are forward 5′-caggcacggtggctcaagcct-3′, reverse 5′-tctgccaccctaaggattaaggctgcgg-3′.

***PD-L1* 3′UTR WT/mutant generation and luciferase assay.** The 3′UTR of *PD-L1* was PCR amplified and cloned into pmirGLO vector using the restriction enzymes SacI and XbaI. Primer pairs used: WT *PD-L1* 3′UTR forward 5′-cgagctcgcattggaacttctgatcttc-3′, reverse 5′-gctctagagttatagaggagaccaagcac-3′. The miR-324-5p and miR-338-5p binding sites were mutated in *PD-L1* 3′UTR by nucleotide replacements through site-directed mutagenesis using the megaprimer inverse PCR method. The forward primer comprised the desired mutation and respective reverse primer was used to generate megaprimers. Primer pairs used: miR-324-5p megaprimer forward 5′-gcccgtaaagcataggcaat-3′, reverse 5′-cgtcgatgagcccctcagg-3′; miR-338-5p megaprimer forward 5′-tgaagatgcgccacagtagatgttac-3′, reverse 5′-gttatagaggagaccaagcac-3′. The miR-324-5p megaprimer was used to amplify the WT *PD-L1* 3′UTR plasmid to generate miR-324-5pΔ *PD-L1* 3′UTR plasmid. The double mutant plasmid was generated utilizing a megaprimer mutant for miR-338-5p-binding sites on the miR-324-5pΔ mutant *PD-L1* plasmid background. THP-1, human monocytic cell line obtained from the National Center for Cell Sciences, Pune, India, was used for the transfection studies. Cells were cultured in DMEM (Gibco-Life Technologies) containing 10% FBS (Gibco-Life Technologies). Transient transfection of THP-1 cells with 3′UTR constructs, β-Galactosidase construct and the miRNA mimics were performed as indicated using low m.w. polyethylenimine (Sigma-Aldrich). After 48 h of transfection and desired stimulation, cells were lysed in Reporter lysis buffer (Promega) and assayed for luciferase activity using Luciferase Assay Reagent (Promega) as per the manufacturer's instructions. The results were normalized for transfection efficiencies measured by β-galactosidase activity. O-nitrophenol β-D-galactopyranoside (HiMedia) was utilized for the β-galactosidase assay.

Statistical analysis. Levels of significance for comparison between samples were determined by the Student's *t* test distribution and one-way ANOVA. The data in the graphs are expressed as the mean ± S.E for values from 3 independent experiments and P values < 0.05 were defined as significant. Graphpad Prism 5.0 software (Graphpad software) was used for all the statistical analysis.

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Author Contributions

S.H. designed and performed experiments, analyzed data, wrote the manuscript. E.S.V. and P.P. performed experiments and analyzed data. M.S., C.S. and V.U. performed experiments. S.V.K. analyzed the data. J.B. and K.N.B. designed experiments, analyzed data, wrote the manuscript and supervised the study.

Additional Information

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Inhibition of programmed death 1 ligand 1 on dendritic cells enhances Mycobacterium-mediated interferon γ (IFN-γ) production without modulating the frequencies of IFN-γ-producing CD4+ T cells.

(Article III)

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Inhibition of Programmed Death 1 Ligand 1 on Dendritic Cells Enhances *Mycobacterium*-Mediated Interferon γ (IFN-γ) Production Without Modulating the Frequencies of IFN-γ–Producing CD4$^+$ T Cells

To the Editor—*Mycobacterium tuberculosis*, the causative agent of tuberculosis, uses several strategies to evade the immune system, which include inhibition of phagosomal maturation and antigen presentation, blockade of apoptosis and autophagy of infected cells, suppression of T-helper type 1 (Th1) and interferon γ (IFN-γ) responses, and expansion of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Tregs) [1–3]. Recently Singh et al reported that *M. tuberculosis* exploits the programmed death 1 (PD-1) pathway to inhibit IFN-γ responses [4]. Conversely, blockade of the PD-1 pathway either by blocking PD-1 on CD3$^+$ T cells or blocking PD-1 ligand 1 (PD-L1) on monocytes in vitro rescued IFN-γ–producing T cells from undergoing apoptosis. However, 2 issues remain unanswered: (1) the specific role of PD-L1 on CD4$^+$ T cells and (2) the contribution of PD-L1 on dendritic cells (DCs), the professional antigen-presenting cells, in polarizing *Mycobacterium*-mediated IFN-γ responses from naive CD4$^+$ T cells.

Human CD4$^+$ T cells, when activated, were reported to express PD-L1 [5]. Therefore, it is likely that interaction of PD-L1–expressing CD4$^+$ T cells with PD-1–positive T cells might modulate IFN-γ responses. We found that *Mycobacterium* induced only a marginal increase in PD-L1 expression on CD4$^+$ T cells (Figure 1*A*). Our results thus indicate that the relatively high expression of PD-L1 on CD3$^+$ T cells (up to 25%) observed by Singh et al [4] upon stimulation with mycobacterial antigens might reflect modulation of PD-L1 expression on CD8$^+$ T cells, rather than CD4$^+$ T cells. PD-L2 expression, however, remained negative on these activated CD4$^+$ T cells (data not shown). In accordance with data on low-level expression of PD-L1 on CD4$^+$ T cells, blockade of this molecule by using monoclonal antibodies (mAbs) did not significantly modulate either the frequency of IFN-γ$^+$CD4$^+$ T cells (Figure 1*B* and 1*C*) or the quantities of IFN-γ secreted from these cells (Figure 1*D*). Thus, our results suggest that PD-L1 on CD4$^+$ T cells plays only a marginal role in mediating impaired IFN-γ responses by *Mycobacterium*.

Dendritic cells (DCs) are sentinels of the immune system that orchestrate primary immune responses to *Mycobacterium* by polarizing distinct CD4$^+$ T-cell responses from naive T cells [1]. Therefore, we next examined the role of PD-L1 on DCs in regulating IFN-γ polarizing responses from naive CD4$^+$ T cells. DCs were generated from circulating monocytes as previously described [6]. Similar to the results obtained with monocytes [4], stimulation of DCs with gamma-irradiated *M. tuberculosis* H37Rv or bacillus Calmette–Guérin induced significant upregulation of PD-L1 (Figure 1*E* and 1*F*). Live *Mycobacterium* bacilli were more efficient in inducing PD-L1 than killed bacilli, implying that, in addition to cell-wall pathogen-associated molecular patterns, secretory antigens and signals associated with replication of bacteria provide stimuli for the induction of PD-L1. However, we could not detect PD-L2 on DCs (data not shown).

Analysis of polarization of T-cell responses from naive CD4$^+$ T cells revealed that so-called *Mycobacterium*-educated DCs significantly enhanced the frequency of IFN-γ$^+$ Th1 cells (Figure 1*G* and 1*H*). However, it was not associated with the increased quantities of IFN-γ secretion from these CD4$^+$ T cells (Figure 1*I*), possibly because of negative signaling by PD-L1 on DCs. Therefore, we attempted to confirm this proposition by blocking PD-L1 on DCs. We confirm that blocking mAbs to PD-L1 were functional, as these antibodies quenched even the basal expression of PD-L1 (Figure 1*E*). Further, in contrast to the results obtained with monocytes [4], blocking PD-L1 on DCs did not significantly alter the frequency of IFNγ$^+$CD4$^+$ T cells (Figure 1*G* and 1*H*). However, PD-L1 blockade led to significant increase in the quantity of IFN-γ produced by CD4$^+$ T cells (Figure 1*I*).

It should be noted that to analyze the expression of surface molecules and intracellular T-cell cytokines, Singh et al stimulated peripheral blood mononuclear cells with *M. tuberculosis* antigens for 48 hours in the presence of brefeldin A, a Golgi transport blocker [4]. For blocking experiments involving PD ligands or receptors, monocyte–T-cell cultures were treated with brefeldin A for 72 hours [4]. However, because brefeldin A is highly toxic to cells if they are treated for longer periods, short-period treatment (duration, typically 4–6 hours) is recommended. Hence, we suggest that the results reported by Singh et al on *Mycobacterium*-mediated IFN-γ responses need to be judged with caution because of the possible toxic effects of brefeldin A.

Together, these results provide insight on how PD-L1 on innate cells regulates IFN-γ responses to *Mycobacterium*. However, the functional repercussion of

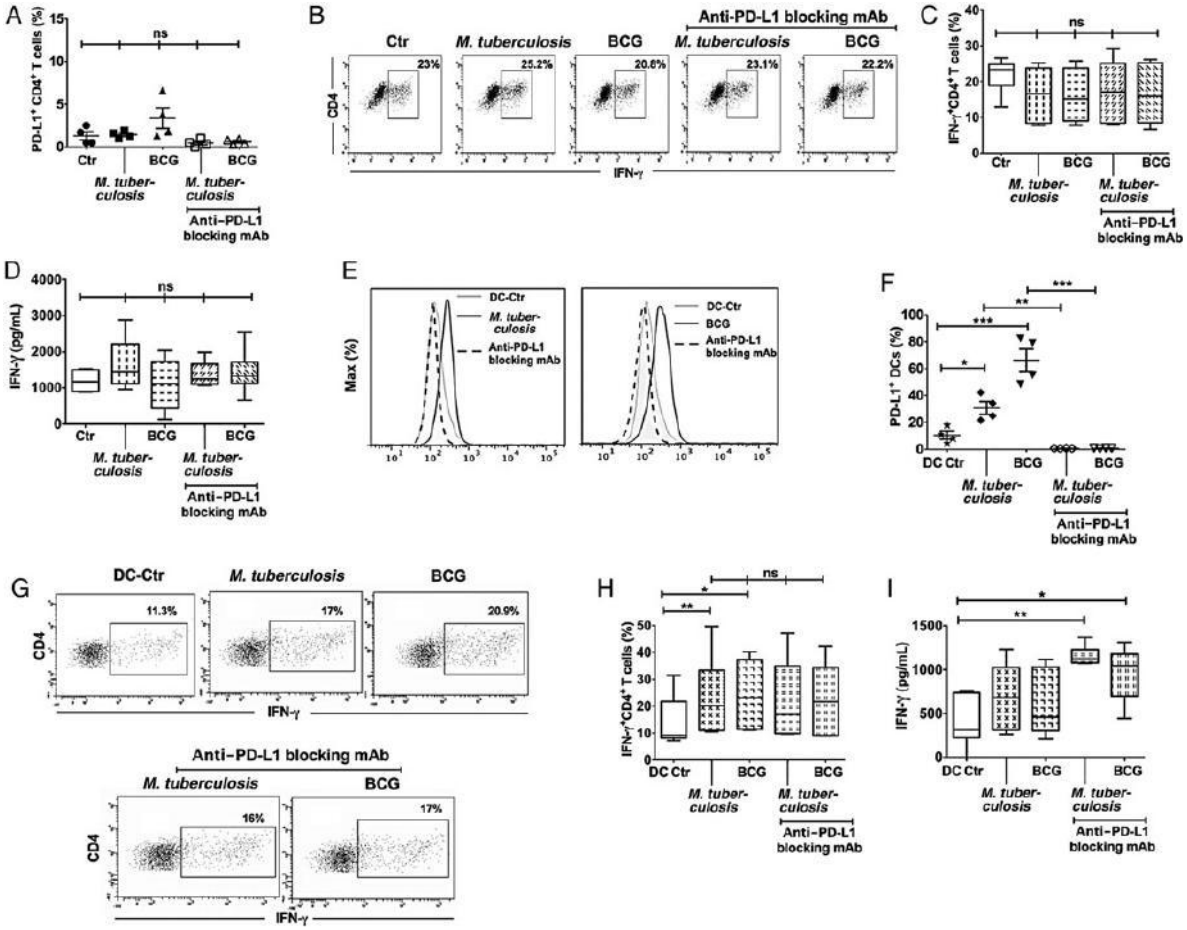

Figure 1. Inhibition of programmed death 1 ligand 1 (PD-L1) on dendritic cells (DCs) enhances *Mycobacterium*-mediated interferon γ (IFN-γ) production without modulating the frequencies of IFN-γ–producing $CD4^+$ T cells. *A*, The expression of PD-L1 on activated $CD4^+$ T cells. $CD4^+$ T cells were isolated from buffy coat specimens from healthy donors by using CD4 microbeads (Miltenyi Biotec, France). Permission from the ethics committee was obtained for the use of buffy coats (protocol 12/EFS/079). $CD4^+$ T cells were cultured in 96-well plates at a concentration of 0.1×10^6 cells/well in 200 µL. Cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs; 1 µg/mL, both from R&D systems, France) alone (Ctr) or with either gamma-irradiated *Mycobacterium tuberculosis* H37Rv (20 µg/mL) or bacillus Calmette–Guérin (multiplicity of infection, 1:10). The expression of PD-L1 was analyzed by flow cytometry (LSR II, BD Biosciences, France) after 5-day culture by using fluorochrome-conjugated mAbs to PD-L1 (BD Biosciences). To block PD-L1 on $CD4^+$ T cells, blocking mAbs to PD-L1 (10 µg/mL, eBioscience, France) were added 18 hours after *Mycobacterium* stimulation. The quenching effect of anti–PD-L1 blocking mAbs was analyzed by flow cytometry. Results are mean (± standard error of the mean [SEM]) for 4 independent donors. *B–D*, The role of PD-L1 on $CD4^+$ T cells in modulating *Mycobacterium*-mediated IFN-γ responses. The $CD4^+$ T cells were cultured and stimulated as described panel *A*. After 5 days, cell-free supernatants were collected, and T cells were activated with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL, Sigma-Aldrich, France), along with GolgiStop (BD Biosciences), for 4 hours. IFN-γ^+CD4^+ T cells were analyzed by flow cytometry. Surface staining was done with fluorochrome-conjugated CD4 mAb (BD Biosciences) and fixable viability dye (eBioscience) to gate and analyze viable $CD4^+$ T cells. Further, cells were fixed, permeabilized (Fix/Perm; eBioscience), and incubated at 4°C with fluorochrome-conjugated mAbs to IFN-γ (BD Biosciences). *B*, A representative dot plot showing the frequency of $CD4^+$IFN-γ^+ T cells. *C*, The results from 6 independent donors are expressed using a box and whisker plot, in which boxes represent the interquartile range of data between the 25th and 75th percentiles, whiskers represent the upper and lower limits of the data, and the dividing line in the box represents the median. *D*, The quantity of IFN-γ (n = 6) in the culture supernatants described above was determined by enzyme-linked immunosorbent assay (ELISA; eBioscience). The results are expressed using a box and whisker plot and the dividing line in the box represents the median. *E* and *F*, The expression of PD-L1 on DCs following stimulation with *Mycobacterium*. Immature DCs (0.5×10^6 cells/mL) derived from peripheral blood monocytes (isolated using CD14 microbeads; Miltenyi Biotec) from healthy donors were cultured in the presence of the cytokines granulocyte-macrophage colony-stimulating factor (1000 IU/10^6 cells) and interleukin 4 (500 IU/10^6 cells; both from Miltenyi Biotec) alone (DC-Ctr) or in the presence of cytokines plus gamma-irradiated *M. tuberculosis* or bacillus Calmette–Guérin for 48 hours. The expression of PD-L1 was analyzed by flow cytometry. Representative histograms (*E*) and mean values (± SEM; *F*) for 4 independent donors are shown. Following *Mycobacterium* stimulation, DCs were incubated with anti–PD-L1 blocking mAbs for 3 hours, and the quenching effect of blocking mAbs was determined by flow cytometry (*E* and *F*). *G–I*, Inhibition of PD-L1 on DCs enhances *Mycobacterium*-mediated IFN-γ production without modulating the frequency of IFN-γ–producing $CD4^+$ T cells. DCs were stimulated *Mycobacterium* and washed extensively. Following incubation with or without anti–PD-L1 mAbs, DCs were cocultured with autologous $CD45RA^+CD25^-$ naive $CD4^+$ T cells (0.1×10^6 cells/well/200 µL) at 1:20 ratios for 5 days. The frequency of $CD4^+$IFN-γ^+ cells was analyzed by flow cytometry. Representative dot blots (*G*) and pooled data for 6 independent donors are expressed using a box and whisker plot (*H*). *I*, The quantity of IFN-γ (n = 6) in the supernatants of DC–$CD4^+$ T-cell cocultures as determined by ELISA. The results are expressed using a box and whisker plot and the dividing line in the box represents the median. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$, by 1-way analysis of variance. Abbreviations: BCG, bacillus Calmette–Guérin; ns, not significant.

PD-L1 blockade might depend on the type of innate cells (monocytes vs DCs) and T cells (memory vs naive T-cell polarization). Previous reports have also implicated PD-L1 in the *Mycobacterium*-mediated expansion of Tregs, the immune suppressor [7, 8]. These data thus provide a rationale for targeting the PD-1–PD-L1 pathway to combat tuberculosis [9, 10].

Notes

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IL-1β but not members of the programmed death pathway is critical for human Th17 response to *M. tuberculosis*

(Article IV)

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IL-1β but not members of the programmed death pathway is critical for human Th17 response to *M. tuberculosis*

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ABSTRACT

The programmed death-1 (PD-1)- programmed death ligand-1 (PD-L1) and PD-L2 co-inhibitory pathway has been implicated in the evasion strategies of *Mycobacterium tuberculosis*. Specifically, *M. tuberculosis*-induced PD-L1 orchestrates expansion of regulatory T cells (Tregs) and suppression of Th1 response. However, the role of PD pathway in regulating Th17 response to *M. tuberculosis* has not been investigated. In the present report, we demonstrate that *M. tuberculosis* and *M. tuberculosis*-derived antigen fractions have differential abilities to mediate human monocyte and dendritic cell (DC)-mediated Th17 response and were independent of expression of PD-L1 or PD-L2 on aforementioned antigen-presenting cells. Importantly, we observed that blockade of PD-L1 or PD-1 did not significantly modify either the frequencies of Th17 cells or the production of IL-17 from $CD4^+$ T cells though IFN-γ response was significantly enhanced. On the contrary, IL-1β from monocytes and DCs were critical for the Th17 response to *M. tuberculosis*. Together, our results indicate that IL-1β but not members of the programmed death pathway is critical for human Th17 response to *M. tuberculosis*

Key words: PD-L1, PD-1, *Mycobacterium tuberculosis*, dendritic cells, monocytes, T cells, Th17, IL-23, IL-1β, FoxP3

INTRODUCTION

Tuberculosis caused by *Mycobacterium tuberculosis* still remains a global threat with an estimated 1.5 million deaths annually. Cellular immunity plays a critical role in mediating the protection against tuberculosis. Indeed IFN-γ-producing $CD4^+$ T helper type 1 cells are critical for the control of *M. tuberculosis* in humans and murine models (1-4). Thus, general paradigm for tuberculosis vaccines has largely focused on enhancing the Th1/IFN-γ response (5, 6). However, despite enhancing IFN-γ response, the recombinant MVA85A vaccine failed to protect infants from tuberculosis (7). Therefore, it is pertinent to decipher the role played by other $CD4^+$ T cell subsets and their cytokines in mediating immunity against *M. tuberculosis*.

Th17 cells that express transcription factor RORC and secrete archetype cytokine IL-17A represent a distinct subset of $CD4^+$ T cells. Th17 cells also produce IL-17F, IL-21, IL-22, GM-CSF and IL-26 and mediate pro-inflammatory responses (8-11). Th17 cells are associated with the pathogenesis of several autoimmune and inflammatory diseases (12-14). Evolutionarily, Th17 response is conserved to mediate protection at mucosal surfaces and against extracellular pathogens (10, 15). Recent reports have also indicated that Th17 response may play a crucial role in mediating protection against intracellular pathogens such as *Francisella tularensis* and *Chlamydia muridarum* (16-18). These data thus indicate the diverse role of Th17 cells in various physiopathologies.

M. tuberculosis employs a plethora of mechanisms to suppress both innate and adaptive immune responses. The role of Th17 response to *M. tuberculosis* is largely pursued in mice and it remains highly controversial (19-25). Recent reports in tuberculosis patients indicate that active disease and its severity are associated with low Th17 response (26, 27). Of note, anti-tuberculosis therapy is associated with enhanced Th17 response, suggesting that *M. tuberculosis* suppresses Th17 response as one of the immune evasion mechanisms (28).

Programmed death-1 (PD-1) – programmed death ligand-1 (PD-L1) and PD-L2 pathway occupies a unique place in the immune evasion strategies employed by *M. tuberculosis*. Recent data highlights the role of PD-1–PD-L1/PD-L2 axis in modulating regulatory T cell (Treg) and Th1 response to *M. tuberculosis* (29-33). Whether this pathway also regulates Th17 response to *M. tuberculosis* is not known. Therefore, in the present study, we have evaluated the role of PD pathway members (PD-L1, PD-L2 and PD-1) in mediating human monocyte and dendritic cell (DC)-mediated Th17 response to *M. tuberculosis*.

Several reports have shown that DCs promote Th17 responses to either *M. tuberculosis* or its antigens (34-37). We found that monocytes and DCs have differential capacity to promote Th17 response to *M. tuberculosis* and *M. tuberculosis*-derived antigens. Notably, a prominent IL-17 response was mediated by DCs in comparison to monocytes. Although both monocytes and DCs did not express PD-L2, PD-L1 was significantly enhanced upon stimulation with *M. tuberculosis*. Similarly, *M. tuberculosis* stimulation of monocyte or DC-$CD4^+$ co-cultures also lead to significant increase in the frequency of PD-1^+CD4^+ T cells. Importantly, blocking PD-L1 or PD-1 neither significantly altered the frequencies of Th17 cells nor augmented IL-17 secretion from $CD4^+$ T cells. Analysis of key Th17 polarizing cytokines indicated that the production of IL-1β was crucial in the establishment of Th17 response to *M. tuberculosis*. These results thus reveal that outcome of Th17 response to *M. tuberculosis* is dictated by the capacity of human innate cells to secrete key Th17 polarizing cytokine (IL-1β) and not expression of members of the PD pathway.

MATERIALS AND METHODS

Antibodies

FITC-conjugated mAbs to CD86 (clone 2331 (FUN-1)), CD274 (Clone MIH1), PE-conjugated mAbs to pSTAT3 (clone 4/P-STAT3), CD80 (clone L307.4), PD-L2 (clone 2D3/B7-H2), APC-conjugated mAbs to HLA-DR (clone G46-6), PD-1 (clone MIH4), Alexa 700-conjugated mAb to CD4 (clone RPA-T4) and BV421-conjugated mAb to CD4 were from BD Biosciences (Le Pont de Claix, France). PE-conjugated mAbs to IL-17A (clone eBio64CAP17), human-mouse RORγt (AFKJS-9), APC-conjugated mAb to FoxP3 (clone 236A/E7) and Fixable Vibility Dye eFluor® 506 were from eBioscience (Paris, France). PE-conjugated mAb to CD40 (clone MAB89) was from Beckman Coulter (Villepinte, France). Blocking mAb to human PD-L1 (clone MIH1) and isotype control mAb were from eBioscience. Alexa-488 conjugated mAb to IL-10 (clone JES59D7) and blocking mAb to PD-1 (clone EH12.2H7) were from Biolegend (London, UK).

M. tuberculosis antigens

γ-irradiated *M. tuberculosis* (strain H37Rv) and *M. tuberculosis* cell wall, cell membrane cytoplasmic fractions were obtained from BEI resources NIAID, NIH.

Purification of immune cells

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags of the healthy blood donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012). Monocytes and autologous $CD4^+$ T cells were isolated from PBMCs by

positive selection using the human CD14 and the CD4 MicroBeads (Miltenyi Biotec (Paris, France) respectively. The cell purity was more than 97%.

Generation of DCs

Monocytes (0.5 x 10^6 cells per ml) were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 IU per 10^6 cells) and IL-4 (500 IU per 10^6 cells) (both cytokines from Miltenyi Biotec) for 5 days to obtain immature monocyte derived DCs (38). The differentiation of DCs was confirmed by flow-cytometry.

Stimulation of monocytes and DCs with *M. tuberculosis* and their fractions

Monocytes or DCs ($0.5x10^6$/ml) were cultured with (20 µg/ml) γ-irradiated *M. tuberculosis* or *M. tuberculosis*-derived cell wall, cell membrane or cytoplasmic fractions (10 µg/ml) for 24 h. Activation of DCs and monocytes was assessed based on the expression of HLA-DR, CD40, CD80 and CD86 by using fluorescence-conjugated mAbs. In addition, induction of PD-L1 and PD-L2 on these cells was also analysed.

Monocyte-$CD4^+$ T cell and DC-$CD4^+$ T cell co-cultures

Monocytes or DCs (10000 cells /200µl/well) were co-cultured with autologous $CD4^+$ T cells at a ratio of 1:10 in U-bottom 96-well plates and stimulated with (20 µg/ml) γ-irradiated *M. tuberculosis* or *M. tuberculosis*-derived cell wall, cell membrane, or cytoplasmic fractions (10 µg/ml) for 5 days. After 5 days, cell-free supernatants were collected, and T cells were activated with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL, Sigma-Aldrich, France), along with GolgiStop (BD Biosciences), for 4 h. For the analysis of IL-17^+ $CD4^+$ T cells and IL-10^+ $CD4^+$ T cells in the co-cultures, surface staining was performed with fluorescence-conjugated MAbs to CD4. Then, cells were fixed, permeabilized using

intracellular staining kit (eBioscience) and incubated at 4°C with fluorescence-conjugated mAbs to IL-17A, IL-10, pSTAT3, and RORC. Samples were acquired by using LSR II (BD Biosciences) flow cytometry and data were analyzed by BD FACS DIVA software (BD Biosciences).

For the analysis of $FoxP3^+$ $CD4^+$ T in the co-cultures, surface staining was performed with fluorescence-conjugated mAb to CD4. Then, cells were fixed, permeabilized using intracellular staining kit (eBioscience) and incubated at 4°C with fluorescence-conjugated mAb to FoxP3.

For the analysis of PD-1 on $CD4^+$ T cells, surface staining was performed with fluorescence-conjugated mAbs to CD4 and PD-1.

PD-L1 and PD-1 blocking experiment

Autologous monocyte-$CD4^+$ T cell and DC-$CD4^+$ T cell co-cultures were stimulated with *M. tuberculosis* for 18 h. Anti-PD-L1 (10 µg/ml), anti-PD-1 (10 µg/ml) or isotype control mAbs were then added to the co-culture. After 5 days, frequency of IL-$17A^+$ $CD4^+$ T cells and IL-17 secretion were analysed.

Validation of role for innate cytokines in *M. tuberculosis*-mediated Th17 responses

DCs (10000 cells /200µl/well) were co-cultured with autologous $CD4^+$ T cells at a ratio of 1:10 in U-bottom 96-well plates and stimulated with (20 µg/ml) γ-irradiated *M. tuberculosis* or *M. tuberculosis*-derived cytoplasmic fractions (10 µg/ml) either alone or in the presence of (10 ng/ml) rhIL-1β (R & D systems, Lille, France) or rhIL-23 (PeproTech, Neuilly-Sur-Seine, France) for 5 days. After 5 days, cell-free supernatants were collected, and T cells were analyzed for Th17 responses by intra-cellular staining as described earlier.

Quantification of cytokines

IL-17A, IFN-γ, IL-6, IL-1β and IL-23 in the cell-free supernatants of monocyte-$CD4^+$ T cell and DC-$CD4^+$ T cell co-cultures were quantified by ELISA (Ready-SET-Go, eBioscience).

Statistical analysis

Statistical analyses were performed by two-way nonparametric Mann-Whitney test or one-way ANOVA (Kruskal-Wallis test or Holm-Sidak's multiple comparisons test) as indicated using Prism 6 software. $P<0.05$ was considered significant.

RESULTS

Human monocytes promote Th17 response to *M. tuberculosis*

Monocytes have been implicated in the establishment of Th17 response in autoimmune diseases (39, 40) and infection (41). We first investigated the ability of human monocytes to promote Th17 response to *M. tuberculosis*. We found that *M. tuberculosis*-stimulated monocytes significantly enhanced both frequency of IL-17A$^+$ CD4$^+$ T cells (**Figures 1A-B**) and the amount of secretion of IL-17A (**Figures 1C)**. On further examination of downstream Th17 signaling events in CD4$^+$ T cells, we found that *M. tuberculosis*-stimulated monocytes significantly enhanced the frequency of pSTAT3 (**Figure 1D**) and RORC (**Figure 1E**). These results thus suggest that human monocytes have the ability to promote Th17 response to *M. tuberculosis.*

Different antigen fractions of *M. tuberculosis* have similar capacity to induce monocyte-mediated Th17 response

M. tuberculosis possesses a plethora of antigens to modulate immune response. Most of these antigens are either located in the cell wall, cell membrane or cytosol. Hence, we investigated whether these different antigens of *M. tuberculosis* have similar or distinct ability to mount monocyte-mediated Th17 response. All these antigenic fractions induced similar level of activation of monocytes as shown by the significantly augmented expressions of CD80, CD86 and CD40 (**Supplementary Figure 1**). Consistent with the activation status of monocytes, all the antigen fractions i.e., cell wall, cell membrane and cytoplasmic fractions significantly enhanced the frequency of IL-17A$^+$CD4$^+$ T cells (**Figure 2A-B**) and the secretion of IL-17A (**Figure 2C**). However, we observed no significant differences in the extent of Th17 response mediated by different fractions of *M. tuberculosis.* Our data thus

indicate that all the antigen fractions of *M. tuberculosis* have similar ability to promote monocyte-mediated Th17 responses.

Dendritic cells differentially promote Th17 response to *M. tuberculosis* and its antigen fractions

DCs play a critical role in mediating protection to *M. tuberculosis* by priming T cell response. Hence we investigated the capacity of DCs to promote Th17 response to *M. tuberculosis* and its antigen fractions. Our results indicate that DCs have the capacity to enhance Th17 response to *M. tuberculosis* (**Figures 3A-C**). However, in contrast to monocytes, human DCs displayed differential ability in stimulating Th17 response to different antigen fractions of *M. tuberculosis*. Thus, cell wall antigen fraction substantially enhanced Th17 response and was comparable to that induced by *M. tuberculosis* bacteria (**Figures 3A-C**). Although cell membrane fraction also enhanced Th17 response, it was lower than that observed with *M. tuberculosis* bacteria and cell wall fraction. Surprisingly, cytoplasmic fraction did not significantly enhance either frequencies of IL-17A$^+$CD4$^+$ T cells or the production of IL-17A (**Figures 3A-C**).

We confirm that low-level induction of Th17 response by cytoplasmic fraction was not because of lack of induction of DC maturation. In fact, DCs stimulated with cytoplasmic fraction significantly enhanced DC maturation markers HLA-DR, CD80, CD86 and CD40 and was similar to those observed with *M. tuberculosis* bacteria, cell wall and cell membrane fractions (**Supplementary Figure 2**)

Further, the inability of cytoplasmic fraction to promote DC-mediated Th17 response was not due to increased frequency of suppressor T cells such as IL-10$^+$CD4$^+$ T cells or FoxP3$^+$CD4$^+$ Treg cells. In fact, the frequency of IL-10$^+$CD4$^+$ T cells remain unaltered upon stimulation with *M. tuberculosis* or its antigen fractions (**Figure 3D**). Consistent with the previous

findings, *M. tuberculosis* and its antigen fractions including cytoplasmic fraction significantly enhanced the frequency of $FoxP3^{+}CD4^{+}$ Tregs (**Figure 3E**). However, the extent of stimulation of Treg response was similar among all three antigen fractions of *M. tuberculosis*.

Differential expression of PD-L1 on monocytes and DCs upon stimulation with *M. tuberculosis* and its antigen fractions

M. tuberculosis employs several strategies to modulate effector CD4 T cell response (42). Several recent reports have indicated that PD-1 – PD-L1/PD-L2 axis has a pivotal role in the regulation of T cell response to *M. tuberculosis* (29, 31, 33). Therefore, to decipher the role of PD-L1/PD-L2 in regulating Th17 response to *M. tuberculosis*, we first investigated the ability of *M. tuberculosis* and its different antigen fractions in modulating PD-L1 and PD-L2 expression on monocytes and DCs. Our results reveal that *M. tuberculosis* bacteria, cell wall fraction and cell membrane fractions significantly induce PD-L1 expression on both monocytes and DCs (**Figures 4A-B**). However, significant induction of PD-L1 by cytoplasmic fraction was observed only in monocytes but not in DCs (**Figures 4A-B**). Unstimulated monocytes and DCs did not express PD-L2 and was not altered upon stimulation with either *M. tuberculosis* or its different antigen fractions (**Figures 4C-D**).

PD-L1 on human innate cells regulates Th1 but not Th17 response to *M. tuberculosis*

PD-1– PD-L1 interaction has been reported to inhibit Th1 response to *M. tuberculosis*. To explore if PD-L1 also checks Th17 response to *M. tuberculosis*, we employed blocking antibodies to PD-L1. We found that blocking PD-L1 either in the monocyte-$CD4^{+}$ T cell co-culture or in the DC-$CD4^{+}$ T cell co-culture did not significantly modify either the frequencies of IL-$17A^{+}CD4^{+}$ T cells or the secretion of IL-17A (**Figures 5A-F**). This data is in line with the fact that lack of induction of PD-L1 on DCs by cytoplasmic fraction of *M.*

tuberculosis was not associated with enhanced Th17 responses (**Figures 3 and 4B**). Whereas, same cytoplasmic fraction promoted Th17 response despite it induced significant expression of PD-L1 on monocytes (**Figures 2 and 4A**). Taken together, these results imply that PD-L1 does not function as negative regulator of Th17 response to *M. tuberculosis*.

It was important to demonstrate that observed phenomenon is not due inefficient PD-L1 blocking. Previous results have demonstrated that PD-L1 negatively regulates IFN-γ responses. Therefore, to demonstrate the efficiency of PD-L1 blocking, we have assessed the secretion of IFN-γ in the monocyte-$CD4^+$ T cell and DC-$CD4^+$ T cell co-cultures. Consistent with the previous data, blockade of PD-L1 significantly augmented the production of IFN-γ from $CD4^+$ T cells (**Figures 6A-B**).

PD-1 is dispensable for the regulation of Th17 response to *M. tuberculosis*

PD-L1 signals via PD-1 on $CD4^+$ T cells and recently it was reported that *Mycobacterium*-induced PD-1 coordinates suppression of Th17 response in tuberculosis patients (28). We confirm that *M. tuberculosis* significantly enhances the frequency of PD-1^+ T cells in the DC/monocyte-$CD4^+$ T cell co-cultures (**Figures 7A and D**). However, blockade of PD-1 did not significantly alter either the frequencies of IL-$17A^+$ $CD4^+$ T cells or the secretion of IL-17A (**Figures 7B-C and 7E-F**). Similar to PD-L1 experiments, blockade of PD-1 also lead to increased IFN-γ responses. Together, these data indicate that human Th17 response to *M. tuberculosis* is not under the control of PD pathway.

IL-1β is critical for mediating Th17 response to *M. tuberculosis*

Upon encountering with pathogens, antigen-presenting cells (APCs) secrete various cytokines that dictate the outcome of T cell response. Particularly, IL-6, IL-1β and IL-23 derived from innate immune cells play a crucial role in establishing and sustaining Th17 response. In

addition, IL-21 and TGF-β also have important role in Th17 priming (8, 14). Thus, to decipher the mechanism of differential Th17 response to *M. tuberculosis* and its fractions by monocytes and DCs, we analyzed the Th17-polarizing cytokines secreted by these innate cells. IL-21 was not produced by monocytes and DCs upon stimulation with *M. tuberculosis* and its antigen fractions. Similarly, basal level of TGF-β was not significantly altered by *M. tuberculosis.* However, upon stimulation with *M. tuberculosis* and its antigen fractions, both innate cells secreted large quantities of IL-6 (**Figures 8A and D**) and moderate amounts of IL-1β (**Figures 8B and E**). Of note, monocytes produced significantly lower amounts (~ 10 fold) of IL-23 as compared to DCs (**Figures 8C and F**). Interestingly, consistent with the lack of modulation of Th17 response, none of these innate cytokines were significantly altered upon blockade of PD-L1 (**Supplementary Figure 3**).

Despite induction of large quantities of IL-6 in DCs similar to those induced by *M. tuberculosis* or its other antigen fractions, lack of Th17 response by cytoplasmic fraction provide a pointer towards dispensability of this cytokine in *M. tuberculosis*-mediated Th17 response. The similar amount of IL-1β production by stimulated monocytes explains elicitation of Th17 response by all the antigen fractions of *M. tuberculosis*. On the contrary, cytoplasmic fraction was inferior to cell wall and cell membrane antigen fractions in promoting IL-1β and IL-23 by DCs and this could explain the lack of Th17 responses observed in DCs upon stimulation with cytoplasmic fraction. Therefore, to establish a role for IL-1β and IL-23 in *M. tuberculosis*-mediated Th17 responses, we supplemented IL-1β or IL-23 to the DC-$CD4^+$ T cell co-cultures that are stimulated with cytoplasmic fraction of *M. tuberculosis*. We found that supplementation of IL-1β enabled cytoplasmic fraction-stimulated DCs to mount strong Th17 response (**Figures 9A-B**), while IL-23 had no effect (**Figures 9C-D**). Thus, our results thus reveal that innate Th17-polarizing cytokine IL-1β but

not members of the PD pathway dictates the outcome of human Th17 response to *M. tuberculosis* and its antigen fractions.

DISCUSSION

M. tuberculosis-loaded aerosols that enter the lungs interact with resident phagocytes which includes alveolar macrophages and DCs. Different subpopulations of DCs are known to co-exist in the human lungs (43). Circulating monocytes form a major reservoir for tissue macrophages and different subsets of DCs (44-46). Moreover monocytes are known to give rise to DCs *in vivo* at mucosal surfaces such as skin (47) and lungs (48, 49), thus suggesting an *in vivo* relevance of monocyte-derived DCs in mediating immune response to mucosal pathogens such as *M. tuberculosis*. In comparison to other infections, there is a delay in the onset of adaptive immune response upon *M. tuberculosis* infection allowing the bacteria to form a niche in the lungs (50). While it is clear that Th1/IFN-γ response is indispensable for the protection against tuberculosis, the role of Th17/IL-17 in mediating protection is unclear.

Initial studies in mice suggested that the IL-23/Th17 axis was dispensable in the overall protection against *M. tuberculosis* challenge, but was required in enhancing the formation of granuloma in the absence of an active IL-12/Th1 axis (51). Surprisingly, Th17 response mediated protection to a highly virulent *M. tuberculosis* strain HN878 by activating macrophages and curtailing bacterial burden in the lungs. However, it was dispensable to the less virulent strains of *M. tuberculosis* thus ascribing a protective role for Th17 to emerging virulent strains (52). Earlier reports in tuberculosis patients indicated that virulent strains such as multi-drug resistant *M. tuberculosis* strongly induce Th17 response. However, enhanced IL-17 was correlated to the severity of the disease and high bacterial burden in the lungs, suggesting a detrimental role for Th17 response in humans (53). More recent reports have demonstrated that tuberculosis patients display lower antigen-specific Th17 response. Incidentally, anti-tuberculosis therapy not only enhanced Th1 response but also augmented Th17 response (28). Furthermore, individuals with bi allelic RORC mutations with a defective Th17 response are associated with a compromised Th1 response and are susceptible

to fungal and mycobacterial infections (54). Thus, it appears that the protective roles of Th17 response may vary depending on the stage of infection. Th17 response contributes to vaccine-mediated protection and during the earlier stages of infection by recruiting lymphocytes and promoting Th1 response. However, chronic exposure or during the later stages of infection it may be detrimental due to neutrophil recruitment that can mediate tissue damage leading to immunopathology.

Previous studies have shown that DCs can mediate Th17 response to *M. tuberculosis* by signaling through TLR-2, dectin-1, DC-SIGN and mannose receptors (36, 52, 55). In the present study, we show that monocytes and DCs have differential capacity to modulate Th17 responses to *M. tuberculosis* and *M. tuberculosis*-derived antigen fractions. Notably, DCs evoked a much stronger IL-17A production from $CD4^+$ T cells than monocytes. This might be due to the capacity of DCs to secrete large quantities of IL-23 than monocytes. IL-23 plays an important role in stabilizing and sustaining the ensuing Th17 response. Interestingly, unlike monocytes that promoted Th17 response to cell wall, cell membrane and cytoplasmic fractions of *M. tuberculosis*, DCs displayed differential response to these antigen fractions. Thus, cell wall fraction triggered strong DC-mediated IL-17A production from $CD4^+$ T cells; cell membrane fraction promoted intermediate IL-17A response and the cytoplasmic fraction did not significantly modulate Th17 response. As immuno suppressor T cells have ability to inhibit effector T cell responses, we surmised whether increased frequency of $IL\text{-}10^+CD4^+$ T cells or $FoxP3^+CD4^+$ Treg cells was responsible for low-level induction of DC-mediated Th17 responses by cytoplasmic fraction of *M. tuberculosis* (56-59). However, $IL\text{-}10^+CD4^+$ T cells were not induced under any stimulatory conditions. Although cytoplasmic fraction significantly enhanced the frequency of $FoxP3^+CD4^+$ Treg cells, this increase was similar to that observed with *M. tuberculosis* and other antigen fractions.

Suppression of cellular immunity reckons a major evasion strategy employed by *M. tuberculosis*. Of the numerous evasion strategies employed by *M. tuberculosis*, the exploitation of PD-L1/PD-L2 – PD-1 axis occupies a central role due to its implication in the expansion of Treg response and suppression of effector Th1 response (60, 61). Considering that induced Tregs and Th17 responses are reciprocally regulated, the possible role of PD-L1/PD-L2 in regulating Th17 response has not been questioned. It is well established that *M. tuberculosis* infection induces PD-L1 on APCs and PD-1 on T cells. Interaction of PD-L1 with PD-1 promotes Treg differentiation and expansion by activating SHP1/2. Induced SHP1/2 suppresses STAT1 functions, abrogates IFN-γ production and abolishes its inhibitory effect on FOXP3 leading to Treg expansion (62). Since PD-1 axis plays a critical role in mediating immune tolerance and loss of which can predispose to inflammatory conditions and autoimmune diseases (63, 64), it is deleterious to completely abrogate PD-L1 – PD-1 signaling during *M. tuberculosis* infection. This is evidenced by the fact that PD-L1-deficient and PD-1-deficient mice are susceptible to *M. tuberculosis* infection and display exacerbated inflammation (65, 66).

In the present study, we found that monocytes stimulated with *M. tuberculosis* and its antigen fractions significantly induce PD-L1 expression on human monocytes. On the contrary only cell wall and cell membrane fractions induced PD-L1 expression on DCs whereas the cytoplasmic fraction failed to enhance PD-L1 on DCs. PD-L2 was not expressed on both monocytes and DCs under any stimulatory conditions. The inability of cytoplasmic fraction to induce PD-L1 on DCs was not due to its lack of stimulatory capacity as cytoplasmic fraction inducted DC maturation similar to that of cell wall and cell membrane fractions.

To decipher the role PD-L1 in regulating Th17 response to *M. tuberculosis*, we employed blocking antibodies to PD-L1. Our results demonstrate that PD-L1 blockade did not significantly alter the frequencies of Th17 cells. On the other hand, IFN-γ production was

significantly enhanced when PD-L1 was blocked, thus confirming the previous data on role of PD axis in regulating Th1 responses (31, 32). However, we did observe a marginal (~50 pg/ml) but insignificant increase in IL-17 production upon PD-L1 blockade. As Th17 cells and Tregs are reciprocally regulated and moreover Tregs are known to suppress Th17 response (65, 67, 68), it is likely that blockade of PD-L1 can indirectly favor Th17 response to a certain extent. This could explain minimal augmentation of IL-17A production that we observed upon PD-L1 blockade.

In addition to PD-L1, *M. tuberculosis* significantly enhanced PD-1 on $CD4^+$ T cells. However, PD-1 blockade had no repercussion on either monocyte- or DC-mediated Th17 response. Our data contradicts a recent report that indicated that *Mycobacterium*-induced PD-1 orchestrates suppression of Th17 response in tuberculosis patients (28). This discrepancy might be due to the fact that previous report focused on active disease where prolonged infection can enhance additional co-stimulatory molecules that might not be present during the earlier phases of stimulation. For example, APCs from active disease express both the co-stimulatory molecules PD-L1 and PD-L2 (31), whereas our current data and previous reports show that DCs from healthy individuals stimulated with *M. tuberculosis* both at the transcript level as well as the protein level induce only PD-L1 and not PD-L2 (30, 33). It is essential to note that we have used γ-irradiated *M. tuberculosis* or *M. tuberculosis*-derived cell wall, cell membrane or cytoplasmic fractions in our experiments and these conditions may not completely mimic *in vivo* situation of infection with live bacteria. Therefore, further work is necessary to decipher the role of PD-1 – PD-L1/PD-L2 pathway in tuberculosis. Nevertheless, our report indicates that PD pathway does not contribute to negative regulation of human Th17 response to *M. tuberculosis*. Also, cytoplasmic fraction of *M. tuberculosis* failed to induce Th17 response despite lack of induction of PD-L1 on DCs.

To decipher the possible mechanism that explains inability of cytoplasmic fraction to promote DC-mediated Th17 response, we analyzed key Th17-polarizing cytokines secreted by monocytes and DCs. Our results indicate that cytoplasmic fraction failed to induce IL-1β, the key cytokine that promotes Th17 response. The amounts of secretion of IL-1β and Th17 responses were significantly correlated. Importantly, exogenous supplementation of IL-1β was sufficient to significantly augment Th17 response by the cytoplasmic fraction. These results indicate that inability of cytoplasmic fraction to induce the production of IL-1β from DCs resulted in its failure to prime a robust Th17 response. Further work on mechanisms underlying the differential production of IL-1β from monocytes and DCs upon stimulation with cytoplasmic fraction is warranted (69). Distinct expression of pattern-recognition receptors that sense cytoplasmic fraction on monocytes and DCs might have resulted in the differential IL-1β secretion upon stimulation with the cytoplasmic fraction. Therefore, antigens that come in direct contact with innate immune cells such as the cell wall antigens are attractive candidates for future tuberculosis vaccines. It is important to note that IL-1β is a critical mediator of immunity to *M. tuberculosis* (70, 71). Our results suggest that this function of IL-1β is in part via enhancement of Th17 responses. As tuberculosis patients were reported to exhibit lower antigen-specific Th17 response (28), supplementing IL-1β might enhance Th17 response.

Taken together, our report demonstrates that DCs have differential capacity to mediate Th17 response to various antigen fractions of *M. tuberculosis*. Therefore, cell wall antigens of *M. tuberculosis* constitute potential subunit vaccine candidates. Importantly we demonstrate that PD pathway is critical for regulating Th1 but not Th17 response to *M. tuberculosis*. IL-1β however is necessary for promoting a prominent Th17 response to *M. tuberculosis*.

Author Contributions

ESV and JB designed the research. ESV, VS, MD, AK, CS, ML and CG performed the research. ESV, VS, MD, AK, CS, ML, CG, SVK and JB contributed to data analyses and data interpretation. ESV and JB wrote the manuscript. ESV, VS, MD, AK, CS, ML, CG, SVK and JB revised the manuscript critically for important intellectual content and approved the final version.

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Figure Legends

Figure 1. Human monocytes stimulated with *M. tuberculosis* enhance Th17 response. Human peripheral blood monocytes were co-cultured with autologous $CD4^+$ T cells at a ratio of 1:10 in X-vivo medium for 5 days with or without γ-irradiated *M. tuberculosis* (Mtb). Th17 cells were analysed by flow-cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A, pSTAT3 and ROR-γt. IL-17A in the cell-free supernatants was quantified by ELISA. **(A-B)** Representative dot plots showing the frequencies of $CD4^+IL\text{-}17A^+$ T cells and **(B)** median ± SEM data from 8 donors. (**C**) The amount of secretion of IL-17A (median ± SEM, n=8). (**D**) Percentage of $CD4^+$ T cells positive for pSTAT3 (median ± SEM, n=4). (**E**) Percentage of $CD4^+$ T cells positive for ROR-γt (median ± SEM, n=4). *, $P < 0.05$; *** $P < 0.001$; as determined by Mann-Whitney t test.

Figure 2. Different antigen fractions of *M. tuberculosis* have similar capacity to amplify human monocyte-mediated Th17 response. Monocytes were co-cultured with autologous $CD4^+$ T cells either alone or were stimulated with *M. tuberculosis*-derived cell wall (CW), cell membrane (CM) or cytoplasmic (Cyt) fractions for 5 days. Th17 cells were analysed by flow-cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A. IL-17A in the cell-free supernatants was quantified by ELISA. **(A-B)** Representative dot plots showing the frequencies of $CD4^+$ $IL\text{-}17A^+$ T cells and (**B**) median ± SEM data from six independent donors. (**C**) The amount of secretion of IL-17A (median ± SEM, n=6). *, $P < 0.05$; ** $P < 0.01$; as determined by one-way ANOVA.

Figure 3. Human dendritic cells differentially promote Th17 response to *M. tuberculosis* and its antigen fractions. Human monocyte-derived DCs were co-cultured with autologous $CD4^+$ T cells at a ratio of 1:10 in X-vivo medium alone or with γ-irradiated *M. tuberculosis*

(Mtb) or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM), or cytoplasmic (Cyt) fractions for 5 days. Th17 cells were analysed by flow-cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A. IL-17A in the cell-free supernatants was quantified by ELISA. **(A-B)** Representative dot plots showing the frequencies of $CD4^+$ $IL\text{-}17A^+$ T cells and (**B**) median ± SEM data from seven independent donors. (**C**) The amount of secretion of IL-17A (median ± SEM, n=7). **(D-E)** Frequencies of $IL\text{-}10^+CD4^+$ T cells or $FoxP3^+CD4^+$ Treg cells in DC-T cell co-cultures stimulated with various antigens of *M. tuberculosis* (median ± SEM, n=5). *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant as determined by one-way ANOVA.

Figure 4. Differential expression of PD-L1 and PD-L2 on monocytes and dendritic cells upon stimulation with *M. tuberculosis* and its antigen fractions. Monocytes or monocyte-derived DCs were either cultured alone or stimulated with γ-irradiated *M. tuberculosis*, or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM), or cytoplasmic (Cyt) fractions for 24 h. Surface expressions of PD-L1 (**A, B**) and PD-L2 (**C, D**) were analysed by flow cytometry. (**A, C**) Percentage of monocytes expressing PD-L1 and PD-L2 (median ± SEM, n=5). (**B, D**) Percentage of DCs expressing PD-L1 and PD-L2 (median ± SEM, n=5). ** $P < 0.01$; *** $P < 0.001$; ns, not significant as determined by one-way ANOVA.

Figure 5. PD-L1 on human innate cells is dispensable for regulating Th17 response to *M. tuberculosis*. Monocytes or DCs were co-cultured with autologous $CD4^+$ T cells either alone or with γ-irradiated *M. tuberculosis*. After 18 hours, PD-L1 blocking mAb or isotype control mAb were added and cultures were maintained for 5 days. Th17 cells were analysed by flow-cytometry by combination of surface staining for CD4 and intracellular staining for IL-17A. IL-17A in the cell-free supernatants was quantified by ELISA. (**A-C**) Representative

dot plots showing the frequencies of $CD4^+IL\text{-}17A^+$ T cells **(A),** (median ± SEM, n= 5) of $CD4^+IL\text{-}17A^+$ T cells **(B)**, and amounts of IL-17A production (median ± SEM, n=6) **(C)** in monocyte-$CD4^+$ T cell co-cultures**.** (**D-F**) Representative dot plots showing the frequencies of $CD4^+IL\text{-}17A^+$ T cells **(D),** (median ± SEM, n= 6) of $CD4^+$ $IL\text{-}17A^+$ T cells **(E)**, and amounts of IL-17A production (median ± SEM, n=6) **(F)** in DC-$CD4^+$ T cell co-cultures**.** *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant as determined by one-way ANOVA.

Figure 6. PD-L1 on human innate cells regulates Th1 response to *M. tuberculosis*. Monocytes or DCs were co-cultured with autologous $CD4^+$ T cells either alone or with γ-irradiated *M. tuberculosis*. After 18 hours, PD-L1 blocking mAb or isotype control mAb were added and cultures were maintained for 5 days. Amounts of secretion of IFN-γ (pg/ml) in monocyte-$CD4^+$ T cell co-cultures (**A**) and in DC-$CD4^+$ T cell co-cultures (median ± SEM, n=6) (**B**). *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant as determined by one-way ANOVA.

Figure 7. PD-1 is dispensable for the regulation of Th17 response to *M. tuberculosis.* Monocytes or DCs were co-cultured with autologous $CD4^+$ T cells either alone or with γ-irradiated *M. tuberculosis*. After 18 hours, PD-1 blocking mAb or isotype control mAb were added and cultures were maintained for 5 days. (**A-C**) Frequency of $PD\text{-}1^+CD4^+$T cells (median ± SEM, n=4) (**A**), frequency of $CD4^+IL\text{-}17A^+$ T cells (median ± SEM, n=6) (**B**) and amounts of IL-17A production (median ± SEM, n=6) (**C**) in the monocyte-$CD4^+$ T cell co-cultures. (**D-F**) Frequency of $PD\text{-}1^+CD4^+$T cells (median ± SEM, n=4) (**D**), frequency of $CD4^+IL\text{-}17A^+$ T cells (median ± SEM, n=6) (**E**) and amounts of IL-17A production (median

± SEM, n=6) (**F**) in the DC-CD4$^+$ T cell co-cultures. *, P < 0.05; ** P < 0.01; ns, not significant as determined by one-way ANOVA.

Figure 8. Production of Th17 priming cytokines by human innate cells upon stimulation with *M. tuberculosis* or its antigen fractions. Monocytes or DCs were co-cultured with autologous CD4$^+$ T cells either alone or stimulated with γ-irradiated *M. tuberculosis*, or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM) or cytoplasmic (Cyt) fractions for 5 days. Cell-free supernatants were analysed for the secretion of innate cytokines by ELISA. (**A-C**) IL-6, IL-1β and IL-23 production by monocytes (median ± SEM, n=5) and (**D-F**) IL-6, IL-1β and IL-23 production by DCs (median ± SEM, n=6). *, P < 0.05; ** P < 0.01; *** P < 0.001; ns, not significant as determined by one-way ANOVA.

Figure 9. IL-1β is critical for mediating Th17 response to *M. tuberculosis.* DCs were co-cultured with autologous CD4$^+$ T cells either alone or stimulated with γ-irradiated *M. tuberculosis*, *M. tuberculosis*-derived cytoplasmic (Cyt) fraction, or Cyt fraction in combination with exogenous IL-1**β** (**A, B**) or IL-23 (**C, D**) for 5 days. Frequencies of CD4$^+$IL-17A$^+$ T cells (median ± SEM, n=4-6) **(A, C)**, and amounts of IL-17A production (median ± SEM, n=4-6) **(B, D)** in DC-CD4$^+$ T cell co-cultures**.** *, P < 0.05; ** P < 0.01; ns, not significant as determined by one-way ANOVA.

Supplementary Figure 1. *M. tuberculosis*-derived cytoplasmic fraction enhance co-stimulatory molecules on monocytes. Monocytes (0.5×10^6/ml) were either cultured alone or stimulated with γ-irradiated *M. tuberculosis* (Mtb), or *M. tuberculosis*-derived cell wall (CW), cell membrane (CM) or cytoplasmic (Cyt) fractions for 24 h. (**A-D**) Surface expression of CD80 (Mean fluorescence intensity (MFI), CD86 (% positive cells), CD40 (MFI) and HLA-DR (MFI) (median ± SEM, n=5) was analysed by flow cytometry. ** $P < 0.01$; ns, not significant as determined by one-way ANOVA.

Supplementary Figure 2. *M. tuberculosis*-derived cytoplasmic fraction induce maturation of DCs. DCs (0.5×10^6/ml) were either cultured alone or stimulated with γ-irradiated *M. tuberculosis* (Mtb), or *M. tuberculosis* -derived cell wall (CW), cell membrane (CM) or cytoplasmic (Cyt) fractions for 24 h. (**A-D**) Surface expression of CD80 (Mean fluorescence intensity (MFI), CD86 (% positive cells), CD40 (MFI) and HLA-DR (MFI) (median ± SEM, n=4) was analysed by flow cytometry. *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; as determined by one-way ANOVA.

Supplementary Figure 3. Production of Th17 polarizing cytokines by human innate cells upon PD-L1 blockade. Monocytes or DCs were co-cultured with autologous $CD4^+$ T cells either alone or stimulated with γ-irradiated *M. tuberculosis*. After 18 hours, PD-L1 blocking mAb or isotype control mAb were added and cultures were maintained for 5 days. Cell-free supernatants were analysed for the secretion of innate cytokines by ELISA. (**A-C**) IL-6, IL-1β and IL-23 production by monocytes and (**D-F**) IL-6, IL-1β and IL-23 production by DCs (median ± SEM, n=7). *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant as determined by one-way ANOVA.

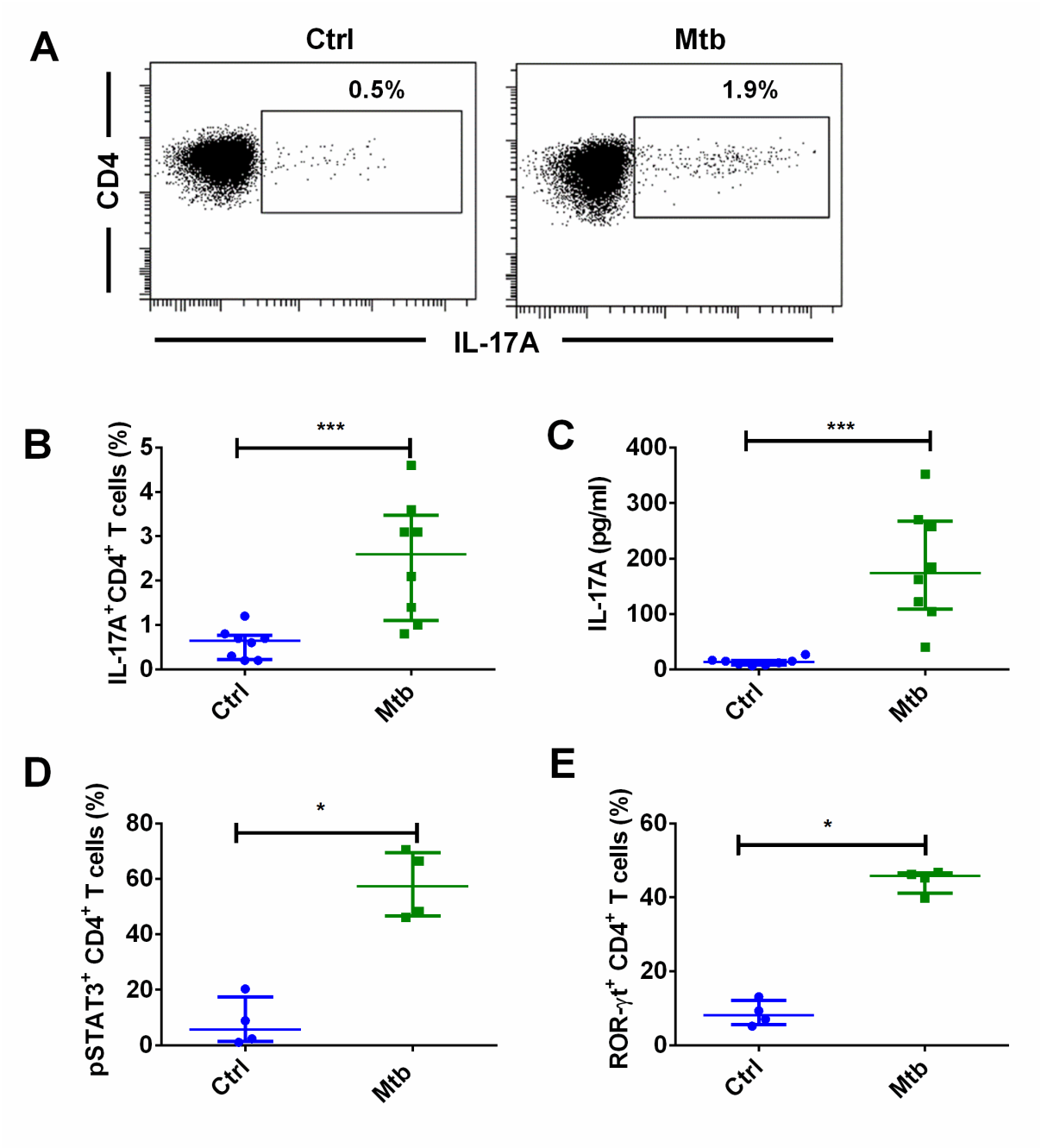
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Figure 1

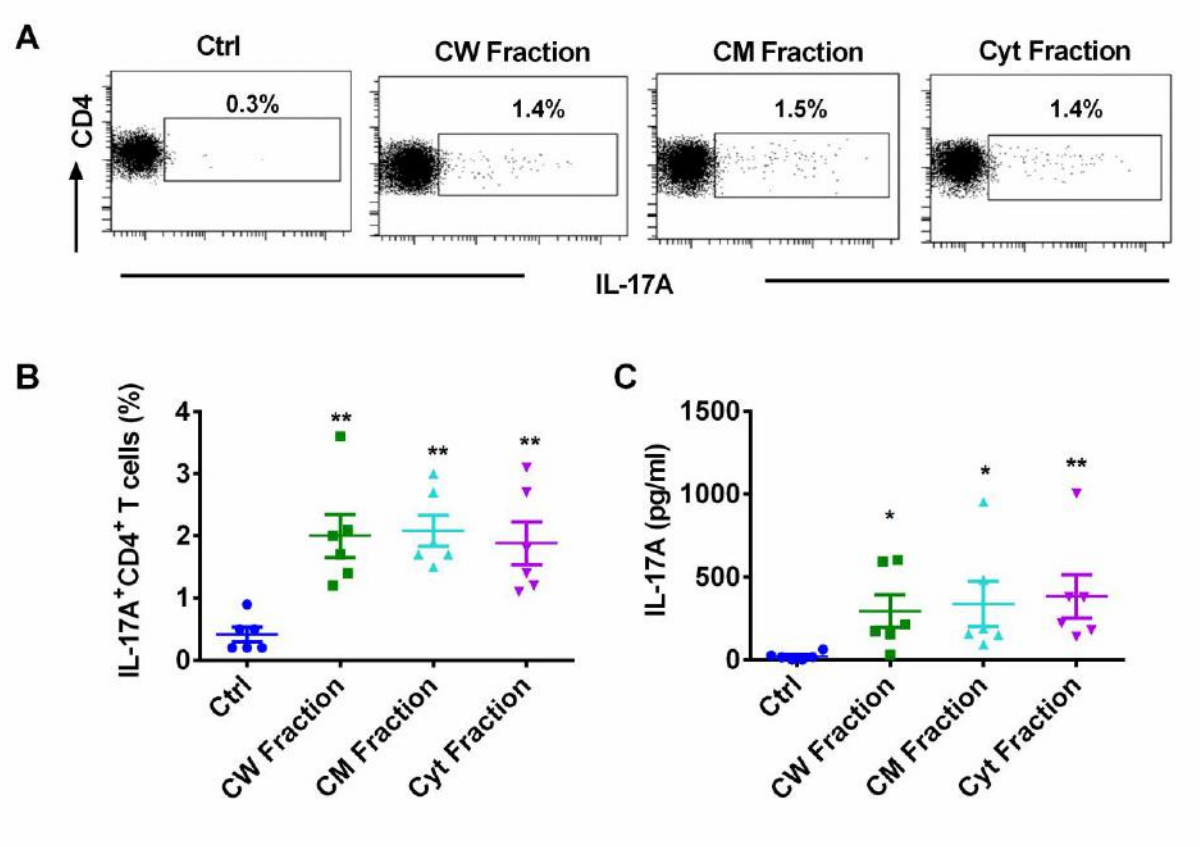
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Figure 2

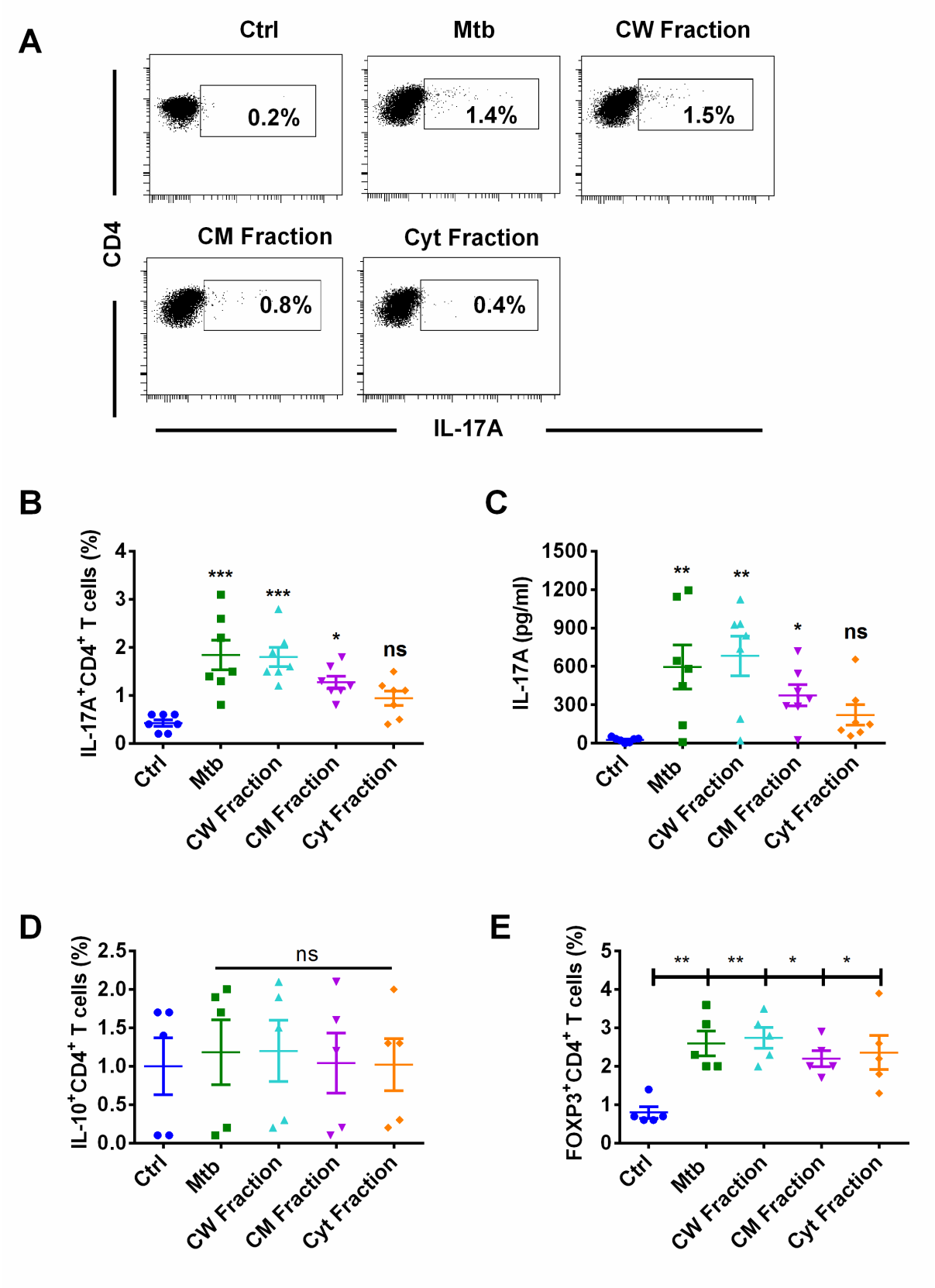
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Figure 3

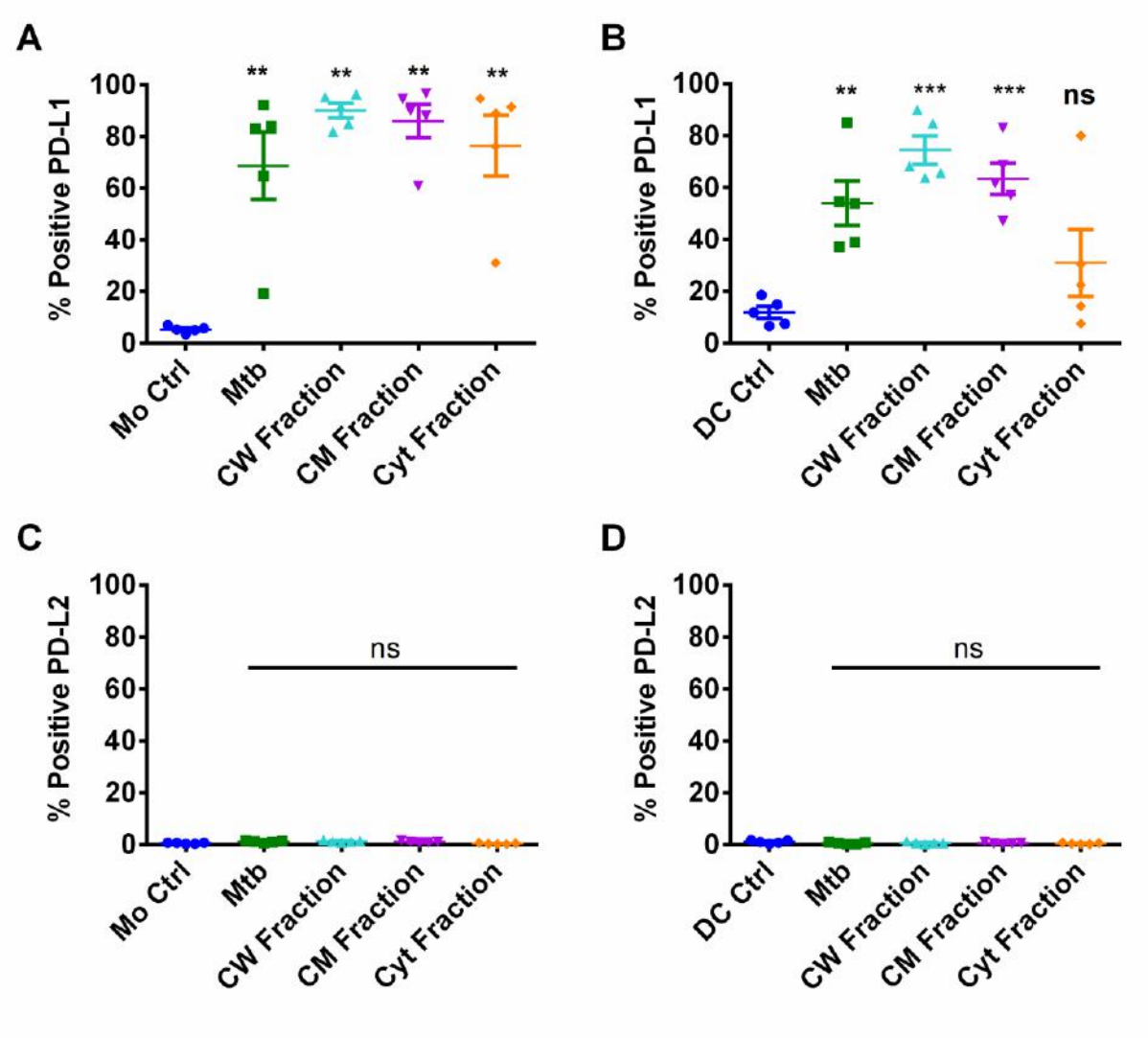
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Figure 4

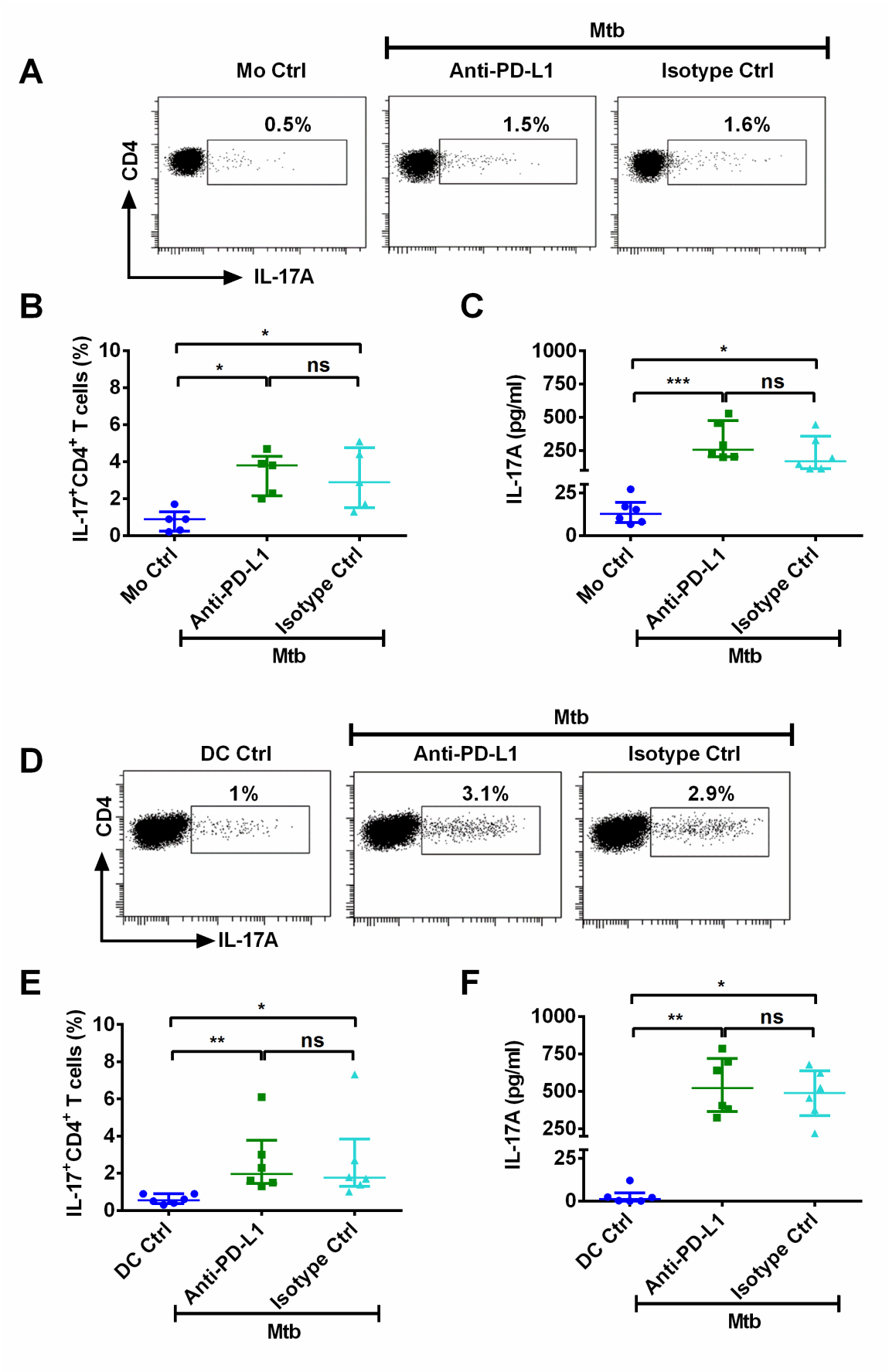
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Figure 5

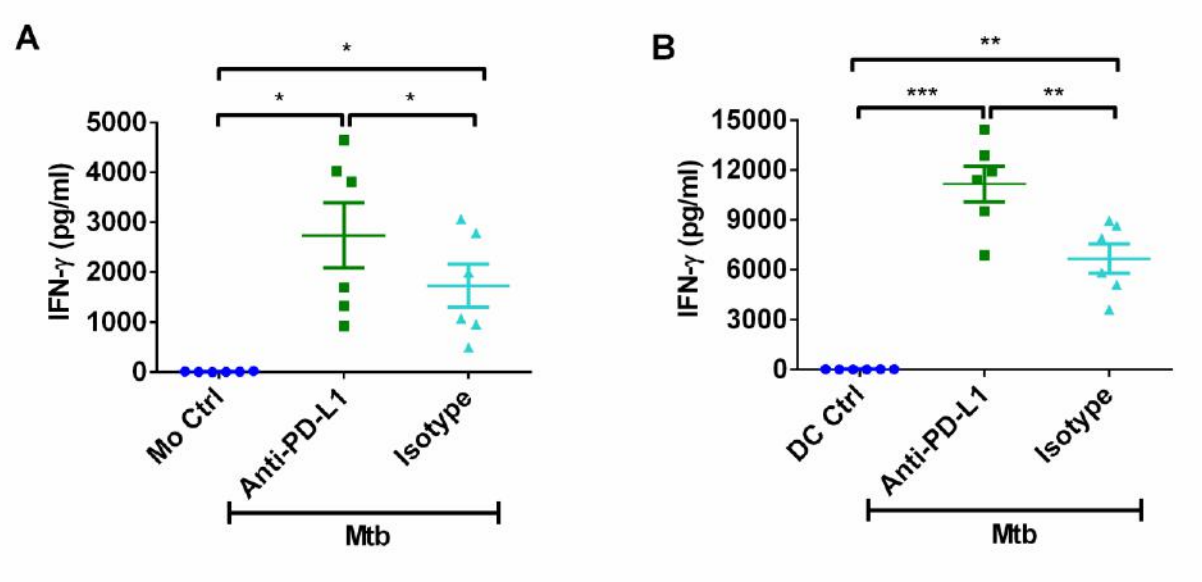
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Figure 6

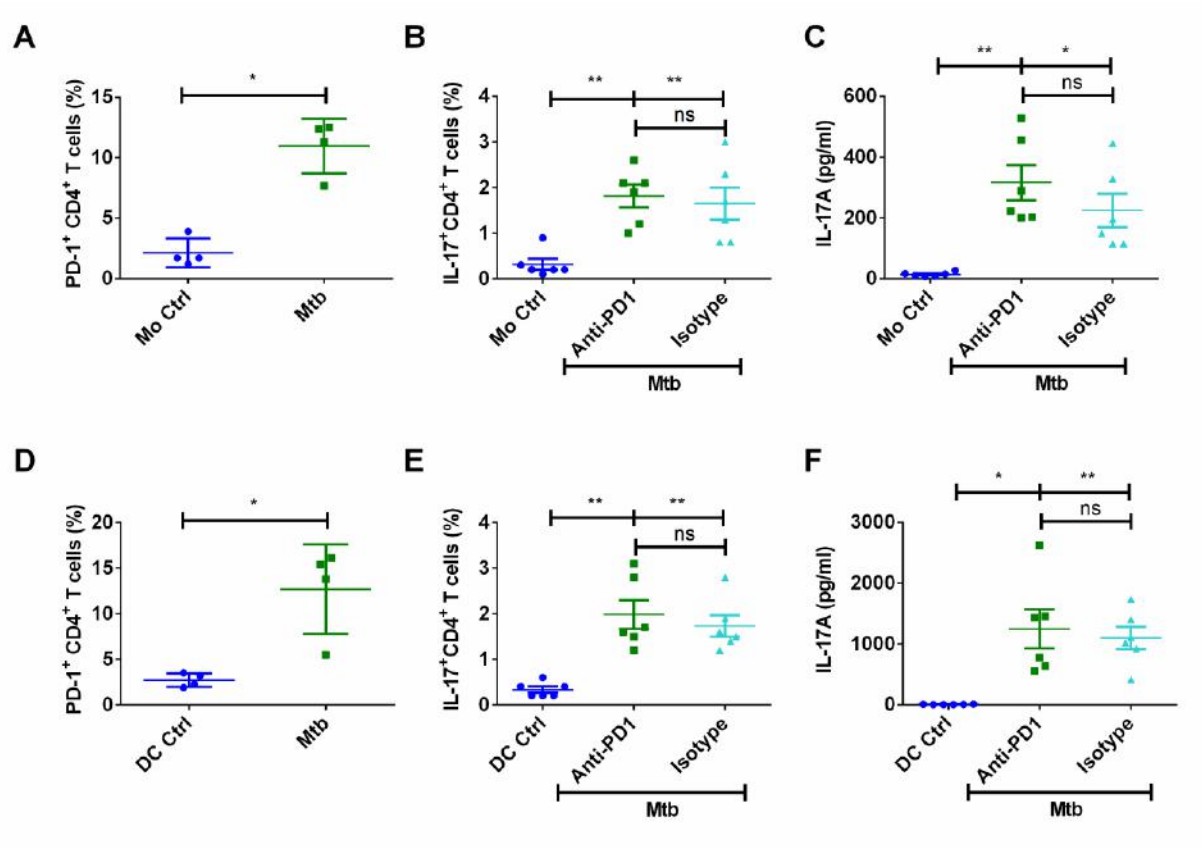
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Figure 7

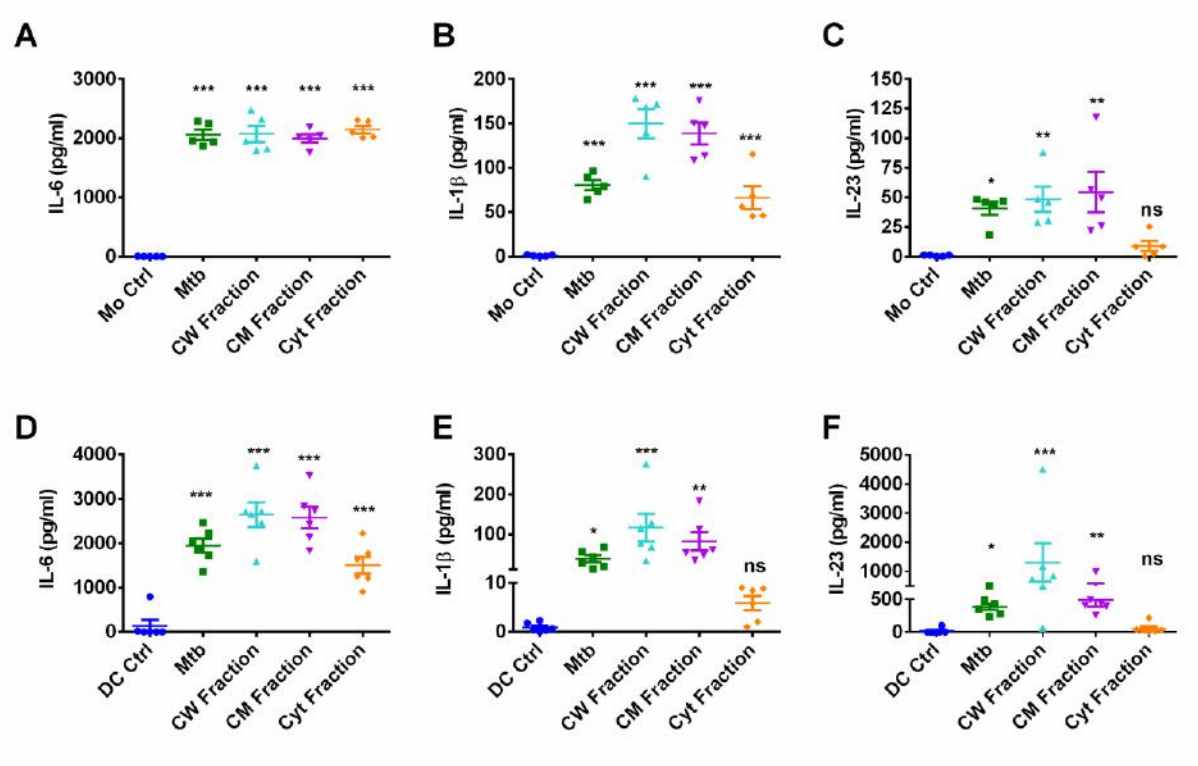
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Figure 8

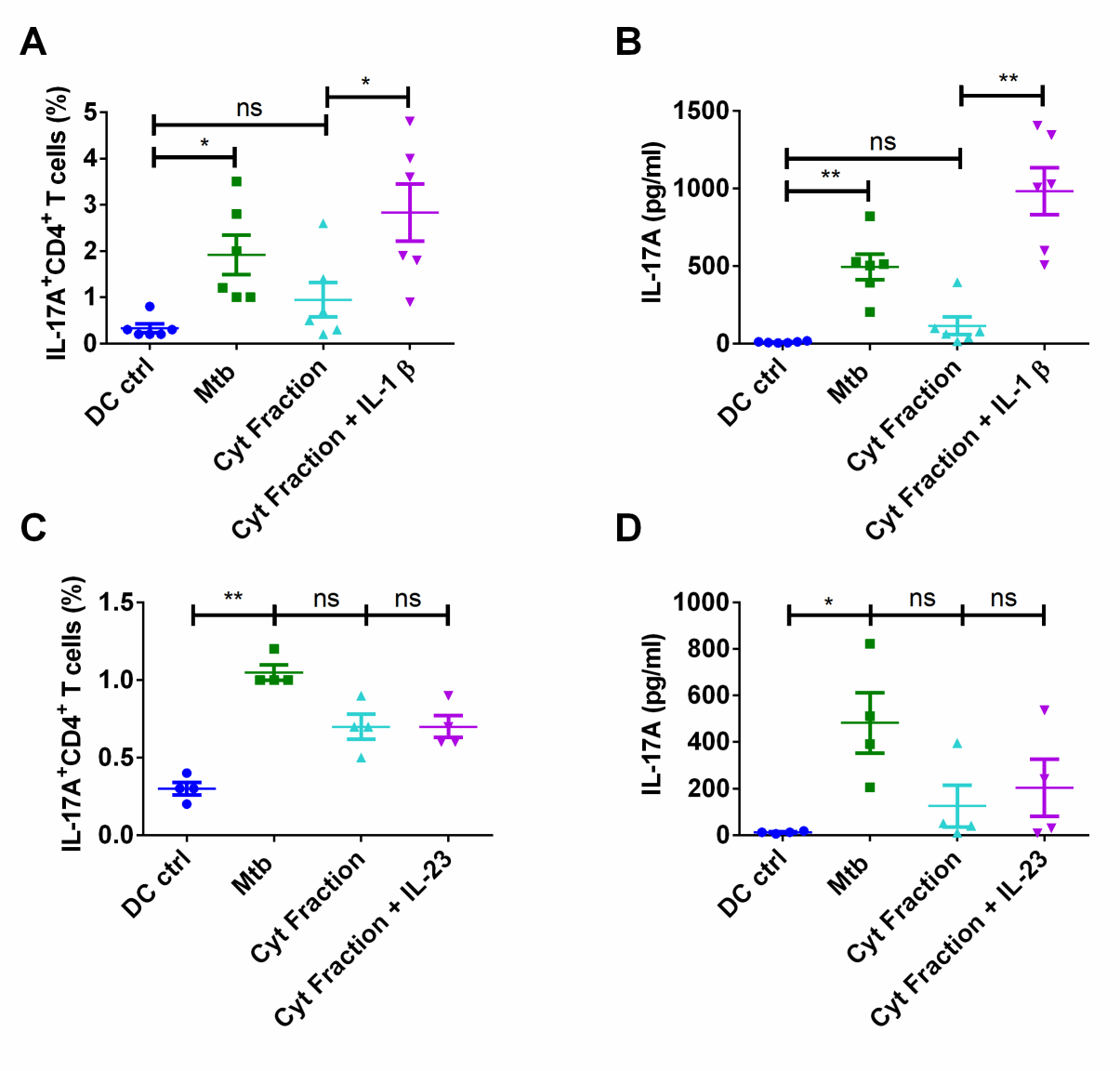
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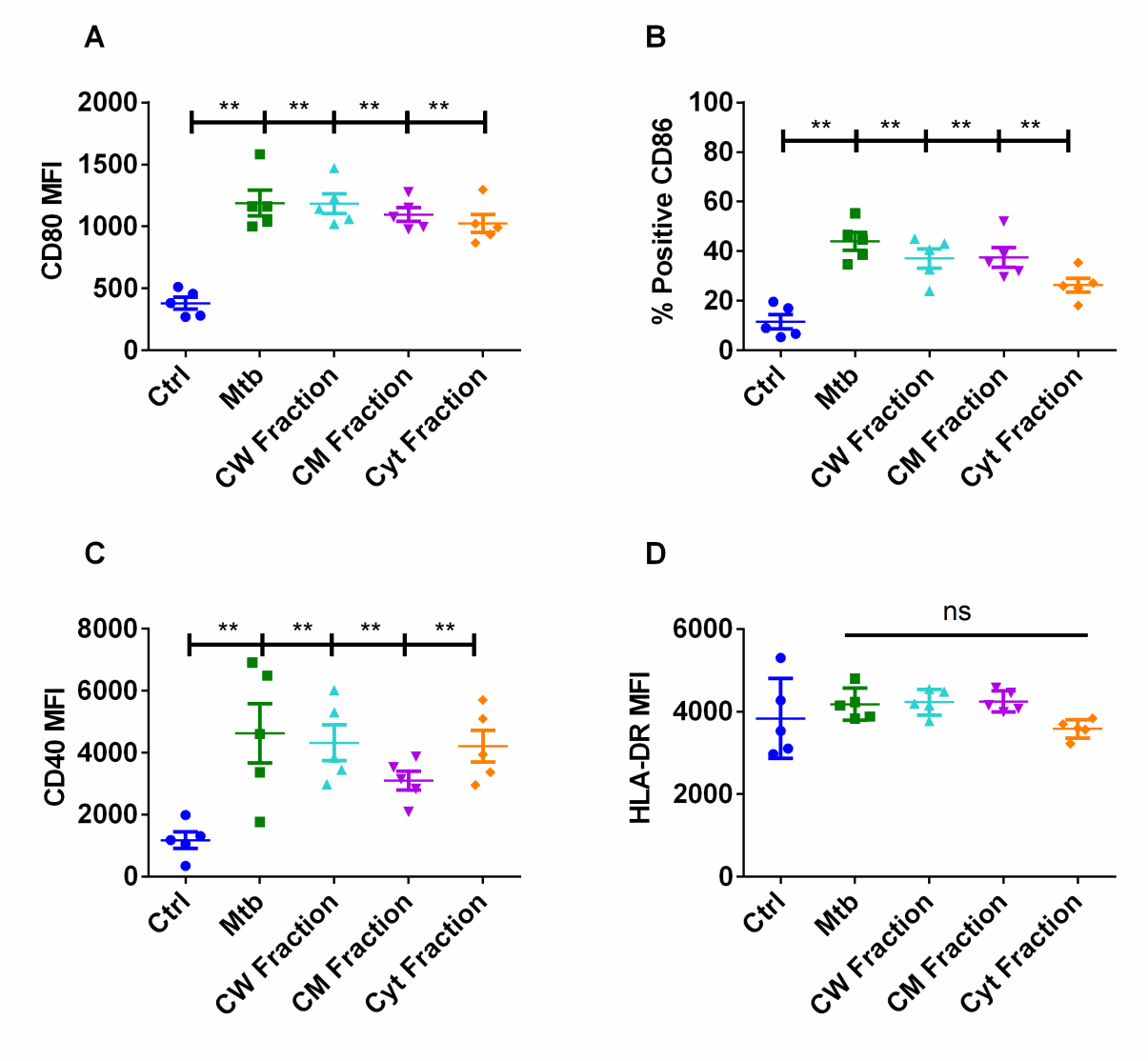
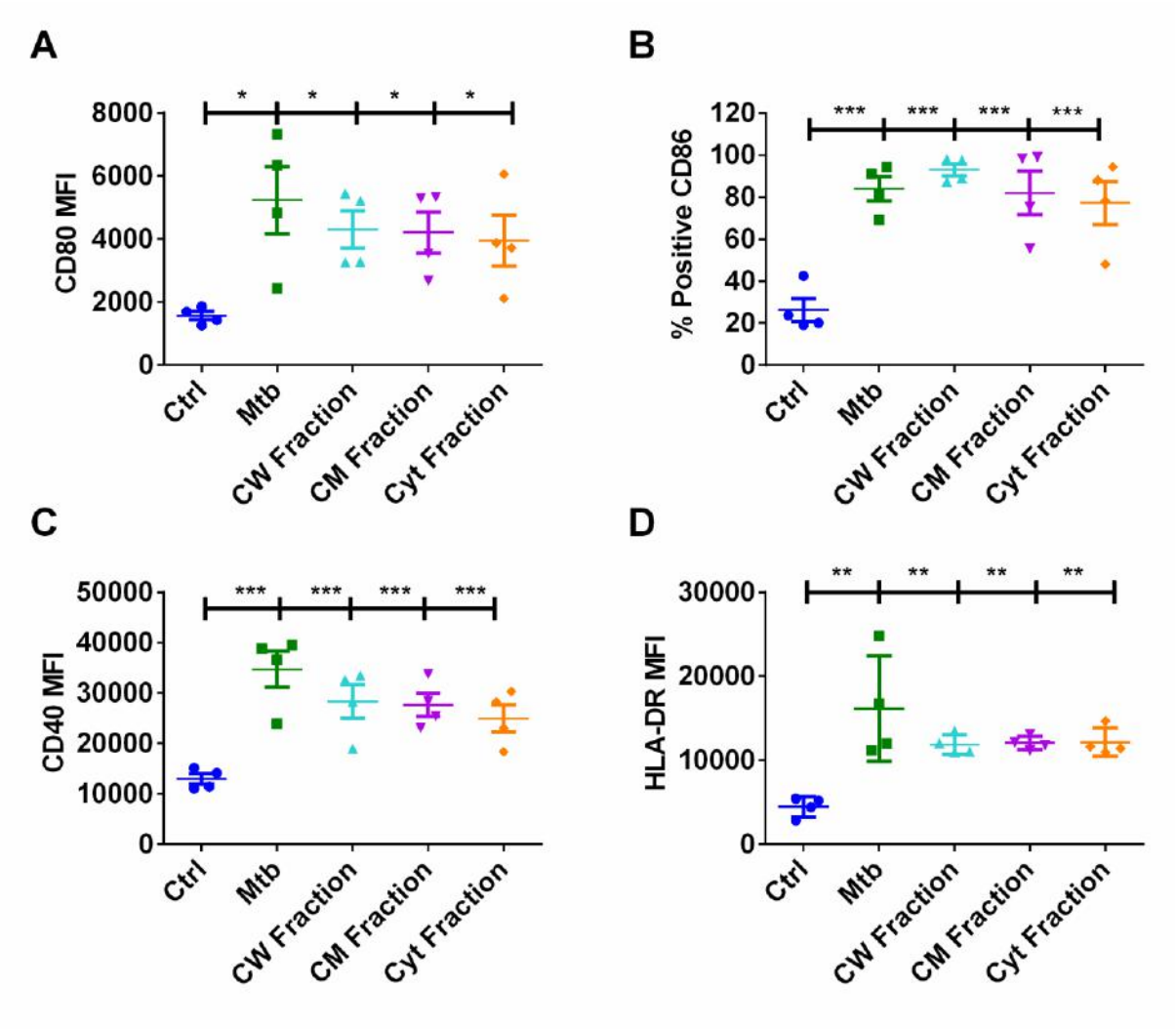
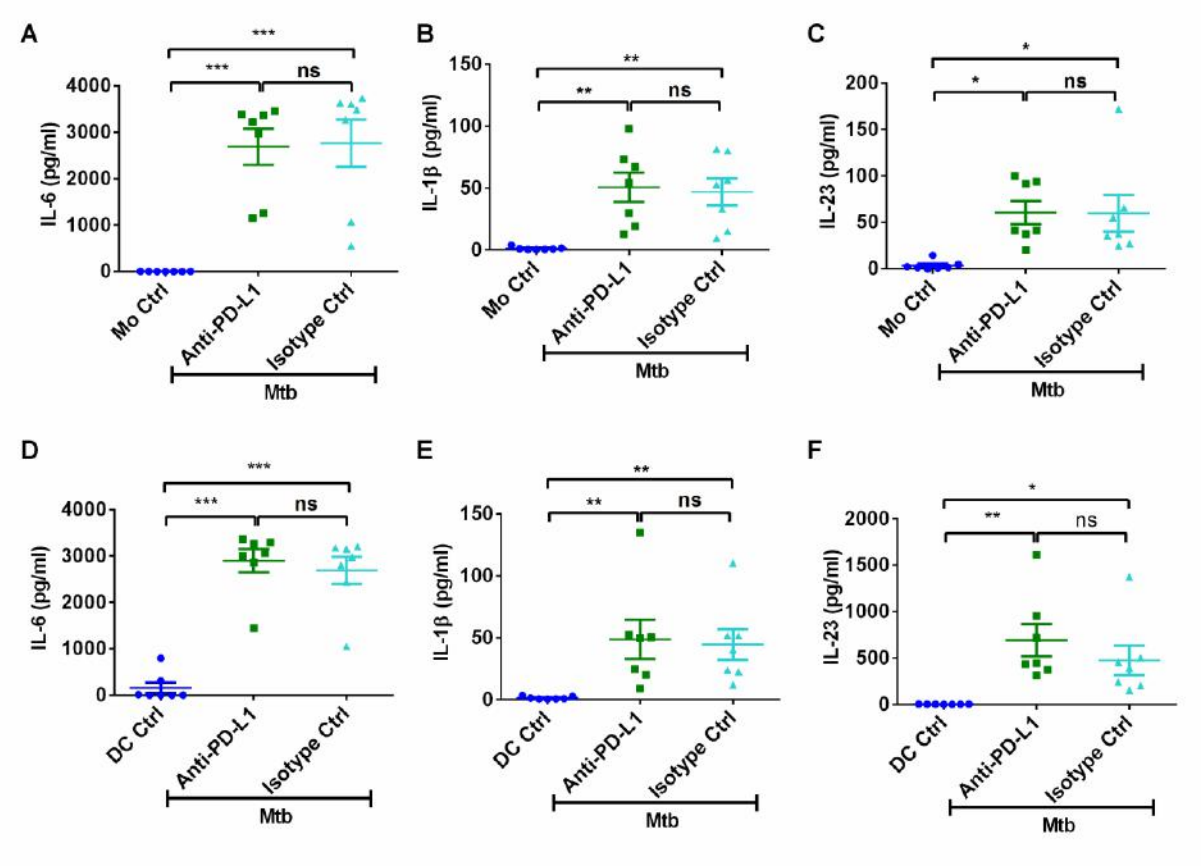
Figure 9



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

Discussion and perspectives

Discussion

Respiratory pathogens that enter the lungs interact with resident phagocytes which include alveolar macrophages and DCs. Different subpopulations of DCs are known to co-exist in the human lungs (van Haarst et al., 1994). Circulating monocytes form a major reservoir for tissue macrophages and different subsets of DCs (Cheong et al., 2010; Leon et al., 2005; Serbina et al., 2008). Moreover monocytes are known to give rise to DCs *in vivo* at mucosal surfaces such as skin (Ginhoux et al., 2006) and lungs (Jakubzick et al., 2008; Osterholzer et al., 2009), thus suggesting an *in vivo* relevance of monocyte-derived DCs in mediating immune response to mucosal pathogens such as *M. tuberculosis* and *A. fumigatus.*

A. fumigatus polysaccharide induce diverse immune response

The surface components of pathogens play a crucial role in the outcome of infection. Thus, most pathogens in addition to harbouring surface components that unveil their presence also harbour components that can dampen immune response and assist in their pathogenesis. The airborne dormant conidia of *A. fumigatus* is composed of a hydrophobic rodlet layer, which conceals underlying polysaccharides, lipids, and proteins. The rodlet layer is an inert protein and thus masks immune recognition. However, conidial swelling and germination remodels the cell wall architecture exposing the concealed polysaccharides. They activate the defense mechanisms of the host producing reactive oxygen-nitrogen intermediates that degrade conidial components (Lapp et al., 2014; Philippe et al., 2003). *A. fumigatus* cell wall β-(1,3)-glucan induces Th1/Th17 responses whereas galactomannan activates Th2/Th17 responses in mice and humans (Bozza et al., 2009a; Rivera et al., 2011). Further, Dectin-1-mediated signaling in murine innate cells enhances Th17 responses to *A. fumigatus* by diminishing IL-12 and IFN-γ (Bozza et al., 2009a; Rivera et al., 2011). GAG inhibits Th1/Th17 cytokine production in human PBMC through the induction of interleukin-1 receptor antagonist (Gresnigt et al., 2014). A recent study with *Candida albicans*-derived chitin demonstrated the secretion of anti-inflammatory cytokine IL-10 in human PBMCs and this IL-10 might be responsible for the lack of induction of Th1 (IFN-γ) and Th17 (IL-17) responses to chitin (Wagener et al., 2014). On the other hand, cell wall chitin from *A. fumigatus* showed both pro- and anti-inflammatory properties; in presence of other PAMPs, chitin showed synergistic pro-inflammatory properties whereas there was the production of anti-inflammatory cytokine IL-1 receptor antagonist in the presence of serum immunoglobulins (Becker et al., 2016). In my

study I found that α-(1,3)-glucan, yet another major cell wall component of the *A. fumigatus*, polarizes human Treg responses. These data thus suggest that cell wall polysaccharides diversify the immune response to *A. fumigatus* by their distinct abilities to polarize specific $CD4^+$ T cell responses.

Dectin-1, TLR-2, and TLR-4 are the major PRRs involved in the recognition of *A. fumigatus*. In my study I found that blocking TLR-2 on DCs prior to stimulation with α-(1,3)-glucan significantly reduced the expression of co-stimulatory molecules on DCs. In line with our observation, *P. boydii* cell wall-derived α-glucan was demonstrated to induce cytokine secretion from mouse peritoneal macrophages and bone marrow-derived DCs in a TLR2-dependent mechanism (Bittencourt et al., 2006); the cytokine secretion was abolished in the macrophages and DCs from TLR2-knockout mice. Further, Chai et al. observed internalization of TLR2 by *A. fumigatus* conidia (Chai et al., 2009). This leads to the hypothesis that, though α-(1,3)-glucan is not an integral component of the conidial surface structure, conidial phagocytosis followed by its attempt to undergo germination inside the phagosome and exposing the cell wall polysaccharides on to the surface as well as conidia-induced TLR2 internalization may be interlinked, which has to be validated. Further, I observed that α-(1,3)-oligosaccharides having dp4-8 and 6-10 are not stimulatory whereas, increase in the chain (dp8-14 and 8-30) enabled them to stimulate DCs. Therefore, the minimum α-(1,3)-oligosaccharides chain-length required to stimulate DCs is in the range of dp10-14. Importantly, the short α-(1,3)-oligosaccharides (dp4-8 and 6-10) efficiently blocked the DC-stimulatory ability of α-(1,3)-glucan. These data fits well with the 'fibril hypothesis' which proposes that soluble short oligosaccharides recognize a single PRR without inducing an immune response, whereas the fibrillar polysaccharides bind to several PRR molecules leading to increased avidity, triggering of signaling events and activation of the immune response (Latgé, 2010).

PD-1 – PD-L1 pathway in host pathogen interaction

In recent years it has become evident that several pathogens of diverse class such as virus, bacteria and protozoans evade the protective immune responses by exploiting PD-L1-PD-1 pathway. Interestingly, the role of PD-1/PD-L1 axis to fungal infections is poorly characterized. Inflammatory sequel during infection can induce PD-L1 on innate cells which upon interaction with PD-1 can supress immune response. In my study, I found that α-(1,3)-glucan and *M. tuberculosis* infection induced substantial expression of PD-L1 on DCs,

however α-(1,3)-glucan did not induce PD-L2. In line with this, previous studies from our lab have demonstrated that *M. tuberculosis* or BCG infection does not induce PD-L2 either at the transcript or at the protein level in monocyte-derived DCs (Holla et al., 2016; Trinath et al., 2012). In contrast, it has been observed that tuberculosis patients have significantly enhanced PD-L2 expression on various APCs (Singh et al., 2013). Therefore, chronic infection under in vivo circumstances may induce additional signals that may trigger PD-L2 expression on innate cells.

The role of PD-1 in suppressing effector T cell response to pathogens was first assessed in a mouse model of LCMV infection. PD-1 was strikingly enhanced on exhausted virus-specific CD8+ T cells, in comparison with functional LCMV-specific CD8+ T cells, suggesting a potential role of PD-1 in curtailing effector T cell function (Barber et al., 2006). Indeed, blockade of PD-1 on these exhausted CD8+ T cells restored effector functions of CD8+ T cells, with enhanced proliferation, cytotoxicity, cytokine production and decreased viremia (Barber et al., 2006). Moreover, as and when the infection is cleared, the expression of PD-1 decreased which also augmented the generation of functional memory T cell population. Subsequent work in different viral infections such as HIV, HBV, and HCV have further supported the negative role of PD-1 in suppressing T cells response during viral infections (Boettler et al., 2006; Day et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). However, although the PD-1 signalling suppresses effector immune response, it plays an indispensable role in monitoring immunopathology. Upon recognition of pathogen innate cells secrete large quantities of proinflammatory cytokines and chemokines which are crucial in protective host response. However, dysregulation of inflammatory response can result in tissue damage and exacerbation of infection. This is supported by the observation that PD-1 and PD-L1 deficient mice are highly susceptible to infection than their wild type counterpart, despite having an enhanced effector T cell functions (Barber et al., 2006; Iwai et al., 2003).

PD-1 – PD-L1 pathway determines balance between Treg and IFN- γ response to α-(1,3)-glucan of *A. fumigatus*

My study suggests that α-(1,3)-glucan-pulsed human DCs polarize predominantly Treg responses. The IFN-γ production was quite low; clear Th1 responses was observed only upon PD-L1 blockade on DCs. Previously it was demonstrated that α-(1,3)-glucan when injected to mice in combination with CpG or upon adoptive transfer of α-(1,3)-glucan–pulsed $CD11c^+$ splenic DC, conferred protection upon challenge with *A. fumigatus* (Bozza et al., 2009b). This

protection was associated with induction of Th1 (IFN-γ) and Treg responses. The discrepancy in the results might be attributed to various factors including vaccination model used in *in vivo* murine experiments, differences in experimental system, differences in the expression pattern of PD-L1 and other PRR, or due to trace amounts of β-(1,3)-glucan with the protocol used to isolate α-(1,3)-glucan. Upon encounter with α-(1,3)-glucan *in vivo*, the final immune response will depend not only on the relative degree of stimulation of particular innate immune cells but also on the diversity of the particular innate cells, cooperation of other innate cells, non-immune cells and PRRs, and the cellular localization.

As discussed earlier, several pathogens evade the protective immune response by exploiting the PD-L1/PD-1 pathway. My results suggests that fungal pathogens such as *A. fumigatus* could also exploit this pathway for immune evasion and persistence. In addition to causing T cell exhaustion and anergy, PD-L1-PD-1 pathway also inhibits IFN-γ responses (Jurado et al., 2008; Sakai et al., 2010a). Of note, PD-L1-PD- 1 pathway blockade leads to enhanced IFN-γ response (Sakai et al., 2010a) and inhibition of Treg expansion (Periasamy et al., 2011; Trinath et al., 2012). Restoration of protective T cell response by blocking PD-1–PD-L1 axis has emerged as an important therapeutic intervention in various forms of cancer including advanced stages of melanoma and non-small cell lung cancer (Brahmer et al., 2015; Larkin et al., 2015). In line with these reports, blockade of PD-L1 on α-(1,3)-glucan-educated DCs lead to enhanced secretion of IFN-γ without the modulation of Th2 and Th17 responses. Th1 response associated with an enhanced production of IFN-γ plays a major protective role in curtailing *A. fumigatus* invasion (Beck et al., 2006; Bozza et al., 2003; Romani, 2011) (8, 58, 59). Importantly, patients with invasive aspergillosis secrete low levels of IFN-γ and administration of IFN-γ, adjunct to antifungal therapy, was associated with significant clinical improvements, contrast to only antifungal therapy with progression in the disease condition(Bandera et al., 2008; Kelleher et al., 2006). Thus my results demonstrate the mechanism by which cell wall components of *A. fumigatus* such as α-(1,3)-glucan could dampen protective immune response against *A. fumigatus* infection.

PD-L1 orchestrates Treg response to *M. tuberculosis*

M. tuberculosis has been shown to employ a range of immune evasion mechanisms including hijacking of PD-1 signalling to modulate effector CD4+ T cell response. Initial studies in murine models of BCG infection suggested that PD-1 signalling had no role in the initial stages of infection, but dampened host immune response during the later stages of

infection. Importantly, the induction of IFN-γ and TNF-α in the WT mice decreased once the PD-1 expression was induced. In line with these observations PD-$1^{-/-}$ mice had enhanced IFN-γ and TNF-α (Sakai et al., 2010b). Moreover, PD-$1^{-/-}$ mice, had significantly lower bacterial burden in the lungs as compared to their WT counterparts during later stages of infection (Sakai et al., 2010b). These results suggest that *Mycobacteria* hijack the PD-1/PD-L1 pathway to suppress effector Th1 response.

In contrast to the above observation, PD-1/PD-L1 signalling was shown to play an essential role during aerosol *M. tuberculosis* infection (Barber et al., 2011). Importantly, CD4+ T cells deficient in PD-1 accelerated *M. tuberculosis* infection. However, CD8+ T cells lacking PD-1 had a minor role. Furthermore, depletion of CD4+ T cells in PD-$1^{-/-}$ mice significantly enhanced the survival of mice as compared to CD4 undepleted PD-$1^{-/-}$ mice (Barber et al., 2011). Interestingly, although PD-$1^{-/-}$ mice had enhanced Th1 response, they had significantly lower Tbet expression (Barber et al., 2011). More recently, it has been demonstrated that enhancing IFN-γ in the lung by about 2 folds in the absence of PD-1 exacerbates rather than control infection. On the contrary enhanced IFN-γ controls infection in the spleen (Sakai et al., 2016). These results thus indicate that the PD-1/PD-L1 signalling regulates Th1-mediated immunopathology during *M. tuberculosis* infection.

Thus, the discrepancies in the final outcome of infection in PD-$1^{-/-}$ mice might be attributed to the infection models used in the study. Further, enhanced Th1 response observed in the PD-$1^{-/-}$ mice was accompanied by a lower Tbet expression. Therefore possible generation of a novel pathogenic subset of Th1 cells might not be ruled out. Importantly, perturbing the PD-1 pathway during infection does not always exacerbate disease. PD-$L1^{-/-}$ mice are susceptible to virulent LCMV clone 13 strain, but not to a less virulent strain of LCMV (Armstrong) (Barber et al., 2006). Further PD-$1^{-/-}$ mice show less signs of disease and rapidly clear the infection upon intranasal *Histoplasma capsulatum* infection, whereas WT mice succumb to disseminated histoplasmosis (Lázár-Molnár et al., 2008). Therefore, the trade-off between pathogen clearance and immunopathology resulting from perturbing the PD-1/PD-L1 pathway will on the target tissue and more importantly, the pathogen in question.

Recently it was demonstrated that *M. tuberculosis* infection enhances PD-L1 not only on APCs but also on CD3+ T cells (Singh et al., 2013). Further, perturbing the PD-1 axis could rescue *M. tuberculosis*-specific IFN-γ producing T cells from undergoing apoptosis (Singh et al., 2013). In contrast, my study suggests that blocking *M. tuberculosis*-induced PD-L1 on DCs

does not significantly alter the frequencies of IFN-γ+ CD4+ T cells, but does significantly augment the production of IFN-γ. This incoherency in my observation might be due to the fact that in the previous study, CD3+ T cells producing IFN-γ was assessed, whereas in my study I have focussed on CD4 T cells. Therefore, it is possible that perturbing the PD-1/PD-L1 axis could have rescued IFN-γ+ CD8+ T cells. Additionally, I found that *M. tuberculosis* induced only a marginal increase in PD-L1 expression on CD4+ T cells which further suggests that the relative high expression of PD-L1 on CD3+ T cells might reflect the expression of PD-L1 on CD8+ T cells.

It has been established that *M. tuberculosis* induces Tregs, which delays the priming of CD4+ and CD8+ T cells in pulmonary draining lymph nodes, thereby delaying the migration of effector T cells to the lungs (Kursar et al., 2007; Shafiani et al., 2010). Further, Tregs in the lungs and granuloma prevent pathogen clearance. Thus, induction and expansion of an inhibitory CD25+ FoxP3+ Treg population reckons as one of the major immune evasion strategies employed by *M. tuberculosis* to combat the protective immune responses. The PD-1/PD-L1 axis has been implicated in the expansion of Tregs during *M. tuberculosis* infection (Periasamy et al., 2011). COX-2-catalysed PGE2 serves as a cue for Treg expansion and functions. PGE2-EP signaling is known to aid in FoxP3 expression (Baratelli et al., 2010; Trinath et al., 2013). Importantly, in humans, mycobacterial infection triggers PGE2-dependent expansion of $CD25^{+}FoxP3^{+}$ Tregs (Garg et al., 2008). My study indicated that mycobacterium-responsive sonic hedgehog (SHH) regulates PD-L1 expression by inhibiting specific microRNAs, miR324-5p and miR-338-5p. Additionally, SHH also induced COX-2, which catalysed the synthesis of PGE-2. Perturbing the PD-1/PD-L1 axis or COX-2/PGE2 axis individually contributed only in part to the expansion of Tregs. However, perturbing of both PD-1/PD-L1 and COX-2/PGE2 significantly abrogated the expansion of Tregs during mycobacterium infection. Interestingly, a recent report showed similar synergistic effects of COX and PD-1 for the eradication of tumors and its usefulness as adjuvants for immune-based therapies.

M. tuberculosis induced Th17 response are independent of PD-1 – PD-L1 axis

Although it well established that $CD4^{+}$ T cells and Th1 cytokines are critical for the control of *M. tuberculosis*, it is also clear that immunity provided by Th1 response alone is insufficient. Therefore, it is pertinent to decipher the role played by other $CD4^{+}$ T cell subsets and their cytokines in mediating immunity against *M. tuberculosis*. Evolutionarily, Th17

response is conserved to mediate protection at mucosal surfaces and against extracellular pathogens (Khader et al., 2009; Meller et al., 2015). However, recent reports have also indicated that Th17 response may play a crucial role in mediating protection against intracellular pathogens such as *Francisella tularensis* and *Chlamydia muridarum* (Bai et al., 2009; Khader and Gopal, 2010; Lin et al., 2009). These data thus indicate the diverse role of Th17 cells in various physiopathologies.

The role of Th17 response to *M. tuberculosis* is highly controversial. Initial studies in mice suggested that the IL-23/Th17 axis was dispensable in the overall protection against *M. tuberculosis* challenge, but was required in enhancing the formation of granuloma in the absence of an active IL-12/Th1 axis (Khader et al., 2005). Surprisingly, Th17 response mediated protection to a highly virulent *M. tuberculosis* strain HN878 by activating macrophages and curtailing bacterial burden in the lungs. However, it was dispensable to the less virulent strains of *M. tuberculosis* thus ascribing a protective role for Th17 to emerging virulent strains (Gopal et al., 2014). Earlier reports in tuberculosis patients indicated that virulent strains such as multi-drug resistant *M. tuberculosis* strongly induce Th17 response. However, enhanced IL-17 was correlated to the severity of the disease and high bacterial burden in the lungs, suggesting a detrimental role for Th17 response in humans (Basile et al., 2011). More recent reports have demonstrated that tuberculosis patients display lower antigen-specific Th17 response. Further, individuals with bi allelic RORC mutations with a defective Th17 response are associated with a compromised Th1 response and are susceptible to Fungal and mycobacterial infections (Okada et al., 2015). Of note, anti-tuberculosis therapy is associated with enhanced Th17 response, suggesting that *M. tuberculosis* suppresses Th17 response as one of the immune evasion mechanisms.

In my study, I found that monocytes and DCs have differential capacity to modulate Th17 responses to *M. tuberculosis* and *M. tuberculosis*-derived antigen fractions. Notably, DCs evoked a much stronger IL-17A production from CD4+ T cells than monocytes. This was due to the capacity of DCs to secrete large quantities of IL-23 than monocytes. IL-23 plays an important role in stabilizing and sustaining the ensuing Th17 response. Interestingly, unlike monocytes that promoted Th17 response to cell wall, cell membrane and cytoplasmic fractions of *M. tuberculosis*, DCs displayed differential response to these antigen fractions. Further, I found that monocytes stimulated with *M. tuberculosis* and its antigen fractions significantly induced PD-L1 expression on human monocytes. On the contrary, only cell wall and cell

membrane fractions induced PD-L1 expression on DCs whereas the cytoplasmic fraction failed to enhance PD-L1 on DCs. This inability of cytoplasmic fraction to induce PD-L1 on DCs was not due to its lack of stimulatory capacity as cytoplasmic fraction inducted DC maturation similar to that of cell wall and cell membrane fractions. Importantly, to decipher the role of PD-L1 in regulating Th17 response to *M. tuberculosis*, I used blocking antibodies to PD-L1. My results suggested that blocking PD-L1 neither significantly altered the frequencies of Th17 cells nor augmented the secretion of IL-17.

My results contradicts a recent report that indicated that *Mycobacterium*-induced PD-1 orchestrates suppression of Th17 response in tuberculosis patients (Bandaru et al., 2014). This discrepancy might be due to the fact that in the previous study, the authors blocked PD-1, which interacts not only with PD-L1 but also with PD-L2. Moreover, the previous report focused on active disease where chronic infection has been shown to induce PD-L2 (Singh et al., 2013). Furthermore, PD-L2 has higher binding affinity to PD-1 than PD-L1 (Ghiotto et al., 2010; Youngnak et al., 2003). Therefore, further work is necessary to decipher the role of PD-L2 in tuberculosis patients. Nevertheless, my report indicates that PD-L1 on APCs does not contribute to negative regulation of human Th17 response to *M. tuberculosis*. Furthermore, cytoplasmic fraction of *M. tuberculosis* failed to induce Th17 response despite lack of induction of PD-L1 on DCs.

To decipher the possible mechanism that explains the inability of cytoplasmic fraction to promote DC-mediated Th17 response, I analysed key Th17-polarizing cytokines secreted by monocytes and DCs. My results indicated that cytoplasmic fraction failed to induce IL-1β and IL-23, two key cytokines that promote Th17 responses. Therefore, my study suggested that PD-L1 is dispensable for mediating Th17 response to *M. tuberculosis* and IL-1β along with IL-23 is necessary for promoting a prominent Th17 response to *M. tuberculosis*.

Conclusion

Taken together, I have demonstrated that *A. fumigatus*-derived α-(1,3)-glucan induces maturation of DCs and secretion of various immunoregulatory cytokines, that was partially dependant on TLR-2. Analysis of $CD4^+$ T cell polarization revealed that α-(1,3)-glucan-educated, DCs predominantly induced CD4+CD25+FoxP3+ Treg generation that was in part dependent on the PD-L1 expression on DCs. Importantly, blocking PD-L1 on DCs enhanced IFN-γ secretion without modulating Th17 response. Similarly, *M. tuberculosis*-induced PD-L1

dampened Th1 response without modulating Th17 response. Analysis of downstream signalling pathways indicated that mycobacterium-SHH mediated the induction of PD-L1 by inhibiting specific microRNAs, miR-324-5p and miR-338-5p that target PD-L1. Additionally, SHH-induced COX-2 catalysed the synthesis of PGE2 that synergized with PD-L1 to coordinate the expansion of Tregs. My results thus demonstrate that respiratory pathogens either directly or by harbouring immunoregulatory antigens highjack the PD-1 – PD-L1 pathway to suppress the protective Th1 response and orchestrate Treg generation without modulating Th17 response. Importantly, my results provide a rational for exploiting immunotherapeutic approaches that target PD-1 – PD-L1 co-stimulatory axis to restore effector T cell response to respiratory pathogens.

Perspectives

In my study, I have demonstrated that *A. fumigatus*-derived α-(1,3)-glucan activates human innate cells in part by signalling through TLR-2. Further, *A. fumigatus*-derived α-(1,3)-glucan imprints regulatory features on DCs which orchestrate induction of Tregs. Mechanistically, α-(1,3)-glucan-mediated induction of Treg is dependent on the expression of PD-L1 on DCs. Although, I observed a synergistic effect between PD-1/PD-L1 signalling and COX-2-induced PGE2 in coordinating Treg expansion by *M. tuberculosis*, α-(1,3)-glucan did not modulate the COX-2 axis (data not shown). Thus, other mechanism involved in the induction of Tregs such as IDO-dependent mechanisms needs to be investigated. Additionally, the potential use of checkpoint inhibitors, in particular PD-1 – PD-L1 axis can be explored in mice models of *A. fumigatus* and in immunocompromised individuals.

Further, human innate response to other cell wall polysaccharides of *A. fumigatus* such as chitin and galactosaminogalactan have not been completely deciphered. Although, it was recently demonstrated that chitin induces both proinflammatory and anti-inflammatory via the Fcγ receptor in human PBMCs, the role of APCs such as DCs and the ensuing CD4+ T cell response remains to be deciphered.

In the context of *M. tuberculosis,* I have demonstrated that *M. tuberculosis*-induced PD-L1 on DCs acts as a negative regulator of protective immune response by inhibiting IFN-γ and expanding Tregs. Importantly, PD-L1 signalling synergizes with COX-2-induced PGE2 to orchestrate Treg expansion. Importantly, I observed that innate cytokines IL-1β and IL-23 but not PD-L1 is indispensable in regulating Th17 response to *M. tuberculosis* and its antigenic fractions.

The utility of COX-2 in enhancing protective immune response can be in murine models of M. tuberculosis infection. Additionally, it is important to note that COX-2 enhances inflammation in the presence of other mediators. Therefore, COX-2-inhibition in addition to reducing Treg response might also reduce inflammatory damage.

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Annexes

PUBLICATIONS

Article 1: **Stephen-Victor E**, Fickenscher H, Bayry J. "IL-26: an emerging proinflammatory member of the IL-10 cytokine family with multifaceted actions in antiviral, antimicrobial and autoimmune responses," **PLoS Pathog (Review).**

Article 2: Maddur MS, Sharma M, Hegde P, **Stephen-Victor E**, Pulendran B, Kaveri SV, Bayry J. Human B cells induce dendritic cell maturation and favour Th2 polarization by inducing OX-40 ligand. **Nat Commun**. 2014 Jun 9;5:4092.

Article 3: Galeotti C, Hegde P, Das M, **Stephen-Victor E**, Canale F, Muñoz M, Sharma VK, Dimitrov JD, Kaveri SV, Bayry J. Heme oxygenase-1 is dispensable for the anti-inflammatory activity of intravenous immunoglobulin. **Sci Rep.** 2016 Jan 22;6:19592.

Article 4: Sharma M, **Stephen-Victor E**, Poncet P, Kaveri SV, Bayry J. Basophils are inept at promoting human Th17 responses. **Hum Immunol**. 2015 Mar;76(2-3):176-80.

Article 5: Sharma M, Schoindre Y, Hegde P, Saha C, Maddur MS, **Stephen-Victor E**, Gilardin L, Lecerf M, Bruneval P, Mouthon L, Benveniste O, Kaveri SV, Bayry J. Intravenous immunoglobulin-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients. **Sci Rep**. 2014 Jul 11;4:5672.

Article 6: Saha C, Das M, **Stephen-Victor E,** Friboulet A, Bayry J,Kaveri SV. Differential effect of Viscum album preparations on the maturation and activation of human dendritic cells and CD4+ T cell response. **Molecules**. 2016 Jul 14;21(7).

PLOS | PATHOGENS

PEARLS

IL-26: An Emerging Proinflammatory Member of the IL-10 Cytokine Family with Multifaceted Actions in Antiviral, Antimicrobial, and Autoimmune Responses

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Cytokines are small proteins that mediate signaling in immune and nonimmune cells, resulting in the modulation of cellular differentiation and activation. These functions are not only important for inflammation but also for antimicrobial responses. Additionally, inflammatory cytokines such as IL-1β and IL-17 can directly interact with microbes and promote their growth [1,2]. In this context, IL-26, an emerging member of IL-10 family cytokines, stands distinct as it exerts antimicrobial response not only by priming various innate immune cells and modulating antiviral responses but also by eliciting direct microbicidal action through affecting the formation of membrane pores.

IL-10 Family Cytokines and Antimicrobial Responses

IL-10 family members play an important role in tissue remodeling and wound healing following infection and inflammation [3]. Growing evidence also suggests an important role for IL-10 family cytokines in mediating and regulating immune response to pathogens. To date, nine cytokines have been categorized into the IL-10 clan, which includes IL-10, the IL-20 subfamily cytokines (IL-19, IL-20, IL-22, IL-24, IL-26), and the distantly related type III interferons (IFN) IL-28A, IL-28B, and IL-29.

IL-10 is a potent anti-inflammatory cytokine that signals through the heterodimeric receptor formed by IL-10R1 and IL-10R2 (Fig 1). By suppressing pro-inflammatory responses elicited by various immune cells, IL-10 limits tissue damage and immunopathology caused during infections [3]. On the other hand, IL-28A, IL-29B, and IL-29 synergize with type I IFNs to support antiviral responses [8].

The IL-20 subfamily cytokines, in general, are vital for innate defense mechanisms at epithelial surfaces. IL-22, the most studied IL-20 subfamily cytokine, regulates epithelial homeostasis and induces antimicrobial agents and β-defensins. IL-22 is produced by lymphocytes and signals through IL-10R2/IL-22R1 heterodimer (Fig 1). IL-22 controls gut microbiota and is involved in the defense against mucosal infections, including those caused by *Candida* and *Klebsiella pneumonia* [4,5]. IL-19, IL-20, and IL-24, collectively known as IL-20 receptor (IL-20R) cytokines, are produced mainly by epithelial cells and myeloid cells and signal through type I IL-20R (IL-20R1 and IL-20R2). IL-20 and IL-24 additionally signal through type II IL-

20R (IL-20R2 and IL-22R1) as well. IL-20R cytokines are critical for tissue homeostasis, epithelial proliferation, and antimicrobial peptide secretion. Recent evidence in a murine model further suggests that IL-20RB-mediated signaling by IL-19 and IL-20 at skin is immunosuppressive and exacerbates cutaneous *Staphylococcus aureus* infection by curtailing IL-1β production and Th17 pathways [6]. Conversely, exogenous administration of IL-24 was reported to modulate IFN-γ responses and neutrophil functions in *Salmonella* infections in vitro and in vivo [7]. The paucity of data warrants further investigations into the precise role of IL-20R cytokines in various infections.

IL-26: An Emerging Member of IL-10 Family Cytokines

IL-26 is an emerging member of IL-10 family cytokines. It was initially named as AK155 following its identification in herpesvirus saimiri-transformed human T cells [9]. Epithelial cells and immune cells, including alveolar macrophages, Th1 and Th17 cells, NK cells, and macrophage-like synoviocytes, are predominant sources of IL-26. The *IL-26* gene is located on chromosome 12q15 and is flanked by genes encoding for two important cytokines, IL-22 and IFN-γ. Secreted IL-26 is a 19-kDa protein containing 171 amino acids with approximately 25% homology and 47% similarity to human IL-10. The protein is largely cationic (~20%), resulting in a positive charge with a calculated isoelectric point of 10.4. IL-26 comprises six helices with a capacity to form dimers and higher-order multimers [9]. IL-26 signals via the heterodimeric IL-20R1/IL-10R2 receptor (Fig 1) and induces Janus kinase-signal transducer and activator of transcription (JAK-STAT) activation, resulting in STAT1 and STAT3 phosphorylation [10,11]. Current evidence suggests that IL-26 recognizes IL-20R1 directly, whereas IL-10R2 helps in the proper assembly of functional IL-26 receptor complex. Although the distribution of IL-10R2 is broad, only a subset of cells, particularly epithelial cells and keratinocytes, expresses IL-20R1 [11]. A recent report shows that myeloid cells, like monocyte-derived dendritic cells, also express mRNA for IL-20R1 [12]. This restricted expression pattern of IL-20R1 limits the action of IL-26.

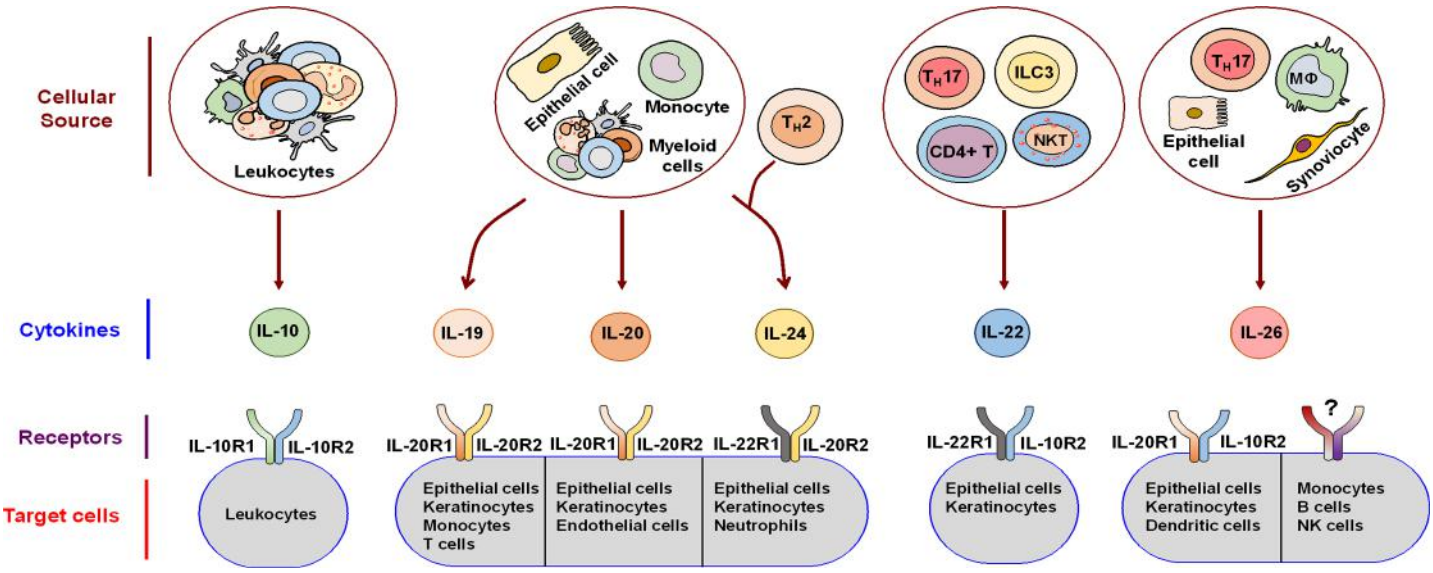

Fig 1. IL-10 family cytokines: source, receptors, and target cells. IL-10 family cytokines are produced by both immune and non-immune cells and signal through heterodimeric receptors expressed on diverse target cells. However, IL-26 might also mediate heterodimeric IL-20R1/IL-10R2 receptor-independent signaling. This scheme was drawn in part by using pictures from Servier Medical Art. NKT, Natural killer T cells; ILC3, Group 3 innate lymphoid cell; MΦ, Macrophage.

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IL-26 also mediates IL-26 receptor-independent signaling as reported in monocytes and B cells [13–15]. This interaction might be mediated via a hitherto unidentified surface receptor or possibly due to the highly cationic nature of IL-26 that facilitates binding to several glycosaminoglycans, including heparin and heparan sulphate, on the cell surfaces [11]. Many functions of IL-26 have only recently been elucidated.

IL-26 Has Diverse Antiviral and Antimicrobial Actions

As expression of IL-20RA, the key subunit of IL-26R that mediates IL-26 signaling, is restricted to skin, intestine, and lungs, it is thought that IL-26 promotes defense mechanisms at mucosal surfaces by bridging immune cells and epithelia. In fact, antimicrobial functions of IL-20 subfamily cytokines, including Th17-derived IL-22, are mediated mainly through the induction of various antimicrobial peptides (i.e., S100A7, S100A8, and S100A9, comprising a group of highly conserved proteins functioning as an immediate line of defense against diverse pathogens) in epithelial cells. However, IL-26 is unique because it does not promote the production of antimicrobial peptides, instead acting as an antimicrobial protein itself.

IL-26 exhibits priming effects on various immune cells in order to boost antiviral and antimicrobial responses. IL-26 induces TNF-related apoptosis-inducing ligand (TRAIL) on human NK cells that kill hepatitis C virus-infected hepatocytes [16]. Furthermore, IL-26 derived from $CD68^+$ alveolar macrophages and Th17 cells propel antimicrobial responses by priming the recruitment of neutrophils towards bacteria and assembled effector immune cells at the lungs, and by triggering the production of plasmacytoid dendritic cell (pDC)-derived IFN-α (Fig 2), respectively [17,18].

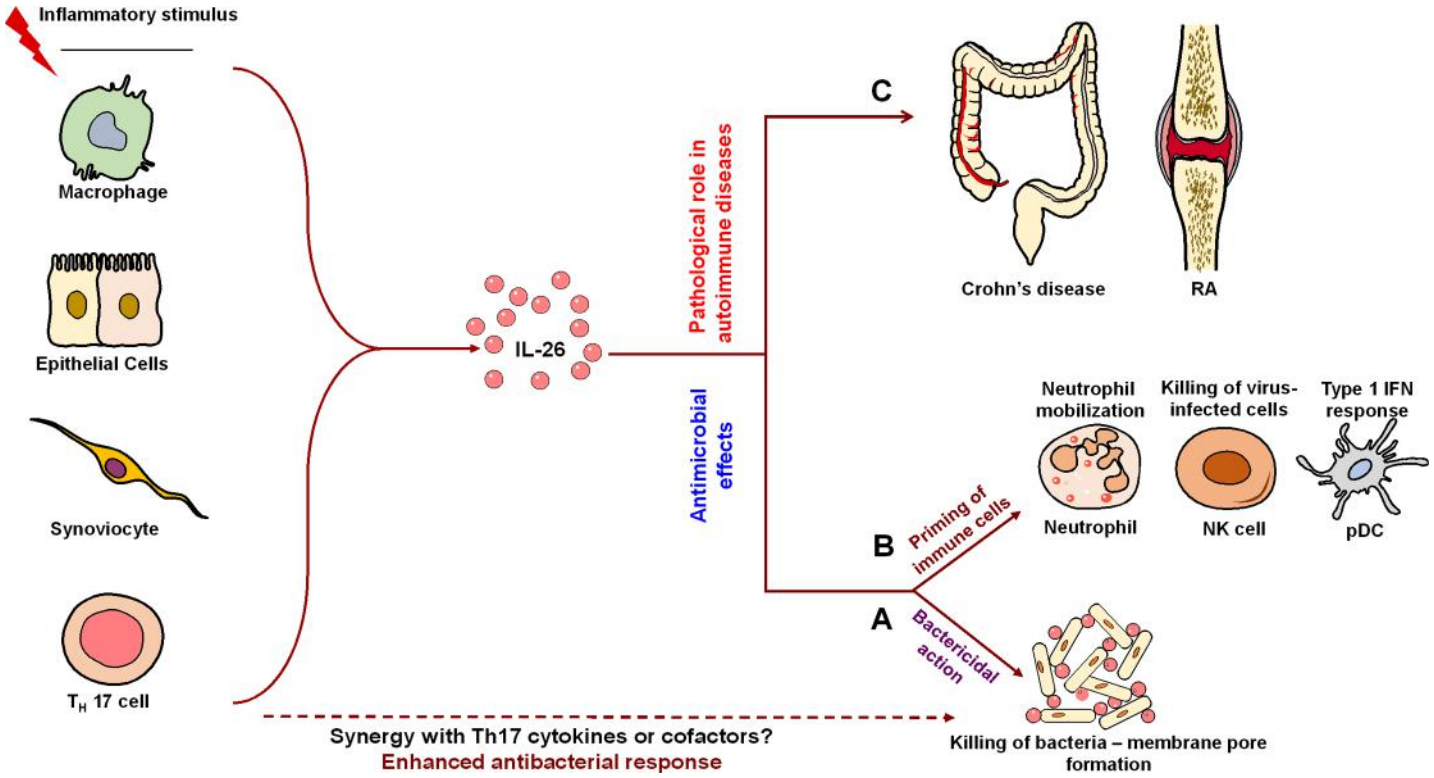

Fig 2. Multifaceted actions of IL-26 in antiviral, antimicrobial, and autoimmune responses. Upon activation, various immune cells secrete IL-26. IL-26 exerts antiviral and antimicrobial actions through dual action by (A) direct killing of bacteria by forming membrane pores and (B) by priming immune cells, such as neutrophils, NK cells, and plasmacytoid dendritic cells (pDCs). Other Th17 cell-derived molecules might act in synergy with IL-26 to enhance this direct killing of bacteria. (C) IL-26 response requires tight regulation as increased expression of IL-26 has been reported in several autoimmune and inflammatory diseases, including Crohn's disease and rheumatoid arthritis (RA). This scheme was drawn in part by using pictures from Servier Medical Art.

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Recent data provide new dimensions to IL-26 functions. Sequence analysis and three-dimensional modeling revealed the cationic amphipathic nature of human IL-26 along with an ability to form multimers, which are similar to other antimicrobial peptides such as defensins. It was reported that human Th17 cell-derived IL-26 mediates protective immunity by direct microbicidal action due to its functional similarity to naturally occurring antimicrobial peptides. When examined, recombinant human (rh) IL-26 inhibited the growth of gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *K. pneumonia*, and gram-positive bacteria like *S. aureus* by direct bactericidal action. The median minimum inhibitory concentration values were in the range of 8.6 to 18.6 μM. IL-26-mediated killing was attributed to the formation of membrane pores and blebs on the surface of bacteria causing membrane disruption and leakage of cytosolic contents (Fig 2) [18]. The bactericidal action of IL-26 was also facilitated in part by its capability to bind lipopolysaccharide and lipoteichoic acid of gram-negative and -positive bacteria with dissociation constants (K_D) of 40.5 ± 4.95 nM and 20.91 ± 2.84 nM, respectively. Local IL-26 concentrations might be considerably increased due to the cationic nature of IL-26 and its affinity to glucoaminoglycans on the cell surfaces [11,15).

IL-26 displays differential abilities in modulating the rate of epithelial and fibroblast cell infections by enveloped viruses. This IL-26R-independent effect was proposed to be largely due to the cationic nature of IL-26, which might accelerate either the binding to or repulsion of viruses from the cells [15].

IL-26 Response Requires Tight Regulation during Infection

It is critical that IL-26 production and signaling be tightly regulated. Chronic infections and their pathological consequences as observed in human lymphatic filariasis are reported to be associated with increased IL-26 expression [19]. IL-26 is up-regulated in the skin and colonic lesions of psoriatic and inflammatory bowel disease patients [20–22]. By binding to its receptors expressed on intestinal epithelial cells, IL-26 inhibits their proliferation and concomitantly induces the production of pro-inflammatory cytokines TNF-α and IL-8 [11,21]. Moreover, increased expression of IL-26 in colonic RORγt-expressing Th17 cells is correlated with the pathogenesis of Crohn's disease (Fig 2) [21]. Similarly, in rheumatoid arthritis, synoviolin$^+$ fibroblast-like synoviocytes and CD68$^+$ macrophage-like synoviocytes constantly secrete IL-26 [13]. Because the aforementioned pathologies have often been suspected to be of infectious etiology [23], these data suggest that once the infection is cleared, IL-26 should be switched-off in order to prevent secondary pathologies. Alternatively, as human IL-26, IFN-γ, and IL-22 are transcribed in the same orientation with related expression patterns and partially common regulatory elements [9,24], it is likely that uncontrolled Th1 and Th17 responses are associated with the IL-26 response in these pathologies.

Therapeutic Use of IL-26 for Bacterial Infections: Future Directions

The newly discovered potent antibacterial action of IL-26 raises the possibility of its therapeutic use for diverse pathogens. However, several key questions remain to be addressed.

First, commensal bacteria were resistant to IL-26-mediated killing [18]. This differential killing suggests that IL-26 could be targeting pathogenic factors that might otherwise be absent in commensals. Identifying the molecular basis underlying the interaction of IL-26 with bacterial components ought to shed light on its specificity and reveal antigenic components that could be exploited for the therapeutic induction of IL-26.

Second, rhIL-26 produced in a prokaryotic expression system was relatively less efficient in killing the bacteria when compared to IL-26 in Th17 cell supernatants. Differences in post-

translational modifications between recombinant and Th17-derived natural IL-26 could be the possible explanations. Comparing antimicrobial activities of rhIL-26 produced in prokaryotic and mammalian expression systems could address this issue. Alternatively, cooperation with other Th17 molecules that act in synergy with IL-26 might not be ruled out and requires further investigations.

Third, the absence of IL-26 in mice has greatly hindered our progress in understanding the antimicrobial functions of IL-26, its regulation, and therapeutic applications for various bacterial diseases. Generating IL-26 transgenic mice might be the solution moving forward.

Nevertheless, the potent bactericidal action of IL-26 raises the possibility of its therapeutic use against selected pathogens, particularly when confronted with antibiotic-resistant bacterial strains. As microbicidal action of IL-26 is independent of other components of the immune system, it also offers a therapeutic option for immunocompromised individuals either alone or in combination with anti-infective immunoglobulins [25,26].

Acknowledgments

Due to space limitations, we have cited only key reports that in no way undermine the great value of uncited studies. We thank Dr. Shriya Raj for the critical comments.

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nature COMMUNICATIONS

ARTICLE

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Human B cells induce dendritic cell maturation and favour Th2 polarization by inducing OX-40 ligand

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Dendritic cells (DCs) play a critical role in immune homeostasis by regulating the functions of various immune cells, including T and B cells. Notably, DCs also undergo education on reciprocal signalling by these immune cells and environmental factors. Various reports demonstrated that B cells have profound regulatory functions, although only few reports have explored the regulation of human DCs by B cells. Here we demonstrate that activated but not resting B cells induce maturation of DCs with distinct features to polarize Th2 cells that secrete interleukin (IL)-5, IL-4 and IL-13. B-cell-induced maturation of DCs is contact dependent and implicates signalling of B-cell activation molecules CD69, B-cell-activating factor receptor, and transmembrane activator and calcium-modulating cyclophilin ligand interactor. Mechanistically, differentiation of Th2 cells by B-cell-matured DCs is dependent on OX-40 ligand. Collectively, our results suggest that B cells have the ability to control their own effector functions by enhancing the ability of human DCs to mediate Th2 differentiation.

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Dendritic cells (DCs) are professional antigen-presenting cells (APCs) and sentinels of the immune system. They perform a prime role in initiating and controlling antigen-specific immune response[1]. These functions of DCs are enabled by their ability to sense and respond to stimuli from their surroundings and to interact with various cells. To perform these functions, DCs transform from an immature state, where they are superior in sensing (antigenic) stimuli, to a mature state, where they are capable of relaying the antigen signatures to adaptive immune cells to induce antigen-specific immune response[1]. DC maturation is characterized by changes in the expression of antigen-presenting molecule HLA-DR, co-stimulatory molecules and cytokine secretion, which influence the outcome of DC interaction with T and B cells[2]. DCs also produce an array of chemokines based on their maturation status to regulate the trafficking of immune cells.

In fact, DCs engage in a cross-talk with different immune cells, including T cells, natural killer (NK) cells and B cells. Reciprocal signalling by these cells can regulate the DC maturation and functions. Accordingly, several studies have shown that T and B cells, innate lymphocytes and neutrophils could influence the quality of immune responses elicited by DCs[1,3–11]. Activated innate lymphocytes and neutrophils induce maturation of DCs with T helper 1 (Th1) polarizing features[8–11]. However, education of DCs by T cells reliant on their subsets: regulatory T cells (Tregs) induce tolerogenic feature on DCs, whereas naive and effector memory T cells induce DC maturation with potent T-cell stimulatory capacity[4–7].

B cells are best known for antibody production. Of note, various reports clearly demonstrated that B cells have profound regulatory functions[3,12–17]. However, only few reports have explored the regulation of DC functions by B cells. Murine models have suggested that B cells might favour the induction of non-polarized immune responses by regulating the functions of DCs[18]. Further, a recent study demonstrated that human B cells that receive signalling via CD40 and Toll-like receptor 9 (TLR9) gain the capacity to restrain the maturation and functions of human DCs[19], although our recent study suggests that regulation of human DCs by B cells depends on the signals they receive[20]. In addition, B cells enhance the production of type I interferon (IFN) by plasmacytoid DCs stimulated with RNA-containing immune complexes[21].

Thus far, only few reports have explored the regulation of human DCs by B cells. Therefore, in the present study we investigated whether B cells could positively regulate human DC maturation and function. We demonstrate that on B-cell receptor (BCR) or CD40-mediated activation, human B cells induce maturation of DCs characterized by enhanced expression of HLA-DR and co-stimulatory molecules CD80, CD86 and CD40. For B cells to exert these effects on DCs, direct cellular contact mediated through molecules associated with B-cell activation such as CD69, B cell-activating factor receptor (BAFF-R) and transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) are essential. Further, activated B cells also induce increased secretion of cytokines and selectively modulate the chemokine production of DCs. These B-cell-matured DCs exhibit enhanced $CD4^+$ T cell (Th) stimulatory capacity with unique features to promote Th2 response without affecting other effector Th cell subsets and Tregs.

Results

BCR-activated human B cells induce maturation of DCs. We first investigated the effect of B cells on phenotype of DCs. Freshly isolated circulating $CD19^+$ B cells, referred as 'resting B cells', were cultured with immature DCs for 48 h (Fig. 1a). We found that resting B cells do not significantly modify the phenotype of DCs ($DC_{Rest\text{-}B}$) and the expression of various DC molecules was similar to that of control DCs (DC_{ctrl}) (Fig. 1b–d). These results indicate that in resting phase, B cells do not provide signals to DCs to undergo maturation. Interestingly, when BCR signalling was provided by $F(ab')_2$ fragments of anti-human IgM antibodies in the DC–B cell co-culture (Fig. 1a), the activated B cells induced maturation of DCs ($DC_{BCR\text{-}B}$). Thus, DCs showed significantly enhanced expression of co-stimulatory molecules CD80, CD86 and CD40, antigen-presenting molecule HLA-DR and terminal maturation marker CD83 (Fig. 1b,c). Consensus with the DC maturation, the expression of antigen uptake receptor CD206 and lipid antigen-presenting molecule CD1a were decreased, whereas the levels of integrin receptor CD11c and C-type lectin receptor DC-SIGN were increased on $DC_{BCR\text{-}B}$ (Fig. 1d). Similar results were observed with allogenic as well as autologous B cell–DC co-culture, suggesting that the changes in DC phenotype are not due to allogeneic reaction.

We confirmed that the maturation of DCs was indeed due to signalling by BCR-activated B cells and not due to nonspecific stimulation of DCs by $F(ab')_2$ fragments of anti-human IgM antibodies used for the BCR activation of B cells. The phenotype of DCs was unaltered when cultured with $F(ab')_2$ fragments of anti-human IgM alone, in the absence of B cells (Supplementary Fig. 1). Further, induction of maturation of DCs by BCR-activated B cells varied directly with the DC:B cell ratio (Fig. 1e). Together, these data demonstrate that BCR-mediated activation license human B cells to induce maturation of DCs.

Induction of DC maturation by B cells is contact dependent. B cells could regulate immune responses both by direct cellular interactions and by secreting various soluble mediators[3,12,19]. Therefore, we explored the mode of interaction involved in the induction of DC maturation by B cells. The B cells were pre-activated with BCR alone ($DC_{pBCR\text{-}B}$) or with BCR + CD40-stimulation ($DC_{pBCR+CD40\text{-}B}$) and immature DCs were co-cultured with these B cells either in direct contact or kept separated in transwells (Fig. 2a). In parallel, DCs were also stimulated with supernatant from activated B cells.

We found that DCs co-cultured in direct contact with activated B cells exhibit phenotypic changes suggestive of maturation, including increased expression of CD83, CD80, CD86, CD40 and HLA-DR (Fig. 2b,c, panel 'Contact'). Interestingly, BCR-mediated activation alone appears to be sufficient for B cells to gain capacity to induce maturation of DCs ($DC_{pBCR\text{-}B}$) as B cells that received additional stimulation via CD40 also induced similar level of maturation of DCs ($DC_{pBCR+CD40\text{-}B}$) (Fig. 2b,c). Furthermore, B cells that received activation signalling via CD40 alone also induced maturation of DCs (Supplementary Fig. 2). Thus, BCR or CD40 signalling enables B cells to induce DC maturation.

In contrast to direct contact, when pre-activated B cells were separated from DCs in transwell plates, DC maturation-associated molecules were not upregulated and DCs retained the phenotype similar to control cells (DC_{ctrl}) (Fig. 2b,c, panel 'Trans'). Even soluble mediators present in the supernatants of activated B cells did not promote maturation of DCs (Fig. 2b,c; panel 'Supernt'). Taken together, these results indicated that direct contact with activated B cells is indispensible and sufficient for the induction of maturation of DCs. Of interest, BCR-pre-activated B cells that were paraformaldehyde fixed also induced maturation of DCs, thus suggesting a role for B-cell surface molecules in the process (Supplementary Fig. 3).

B-cell activation-associated molecules mediate DC maturation. As activation of B cells and contact-dependent interaction

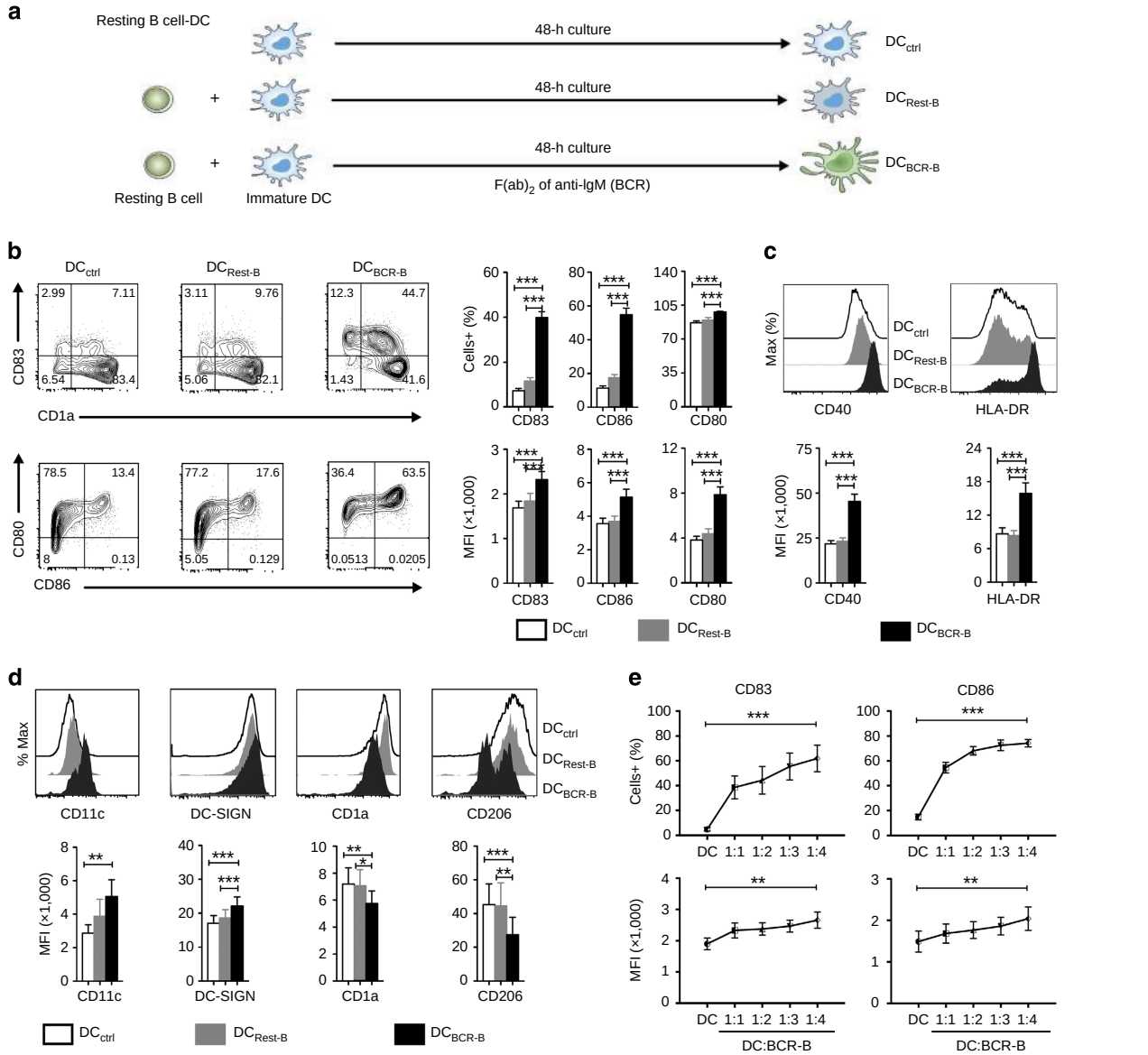

Figure 1 | BCR-activated B cells induce maturation of DCs. (**a**) Experimental design. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC_{ctrl}), or co-cultured at 1:1 ratio with $CD19^+$ B cells that were either in resting phase ($DC_{Rest\text{-}B}$) or directly activated in DC–B cell co-culture via BCR stimuli ($DC_{BCR\text{-}B}$). Phenotypic analysis of CD20-negative cells was done by flow cytometry. (**b**) BCR-activated B cells enhance the expression (% positive cells and mean fluorescence intensity, MFI) of DC activation markers and co-stimulatory molecules. (**c**) BCR-activated B cells upregulate expression of HLA-DR and CD40 on DCs. (**d**) Modulation of expression of CD11c, DC-SIGN, CD1a and CD206 on DCs by BCR-activated B cells. Representative plot and mean ± s.e.m. of data from 10 to 24 donors. (**e**) Immature DCs were co-cultured with increasing number of B cells in the presence of BCR stimuli for 48 h and expression (% positive cells and MFI) of CD83 and CD86 was analysed on CD20-negative cells. Mean ± s.e.m. of data from four donors. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ by one-way analysis of variance test.

between DCs and B cells were necessary for the maturation of DCs, we aimed at identifying the activation-associated B-cell surface molecules implicated in this process. We found that in addition to upregulation of classical B-cell activation-associated molecules, such as HLA-DR, CD25, CD38, CD40, CD80 and CD86, B-cell stimulation also led to significantly enhanced expression of CD69, BAFF-R and TACI (Fig. 3a,b). We found that BCR (B_{BCR}) or BCR + CD40 ($B_{BCR+CD40}$) signalling was superior to CD40 signalling alone in their ability induce the expression of B-cell activation-associated molecules (Fig. 3a,b). As CD69, BAFF-R and TACI could interact with corresponding ligands on DCs, we explored the role of these B-cell surface molecules in the process of DC maturation by employing blocking antibodies.

Interestingly, on blocking TACI, BAFF-R or CD69 individually lead to only minor inhibition of DC maturation. However, blocking all three receptors simultaneously lead to significant inhibition of DC maturation as analysed by the phenotype (Fig. 4). Of note, adhesion molecules CD54 and CD11a/CD18 were found to be not involved in the process. We also found that the expression of CD69 on B cells implicates BCR or CD40 signals received by the B cells but not feedback signals by BAFF as treatment of B cells with BAFF led to only minor changes in the expression of CD69 on B cells.

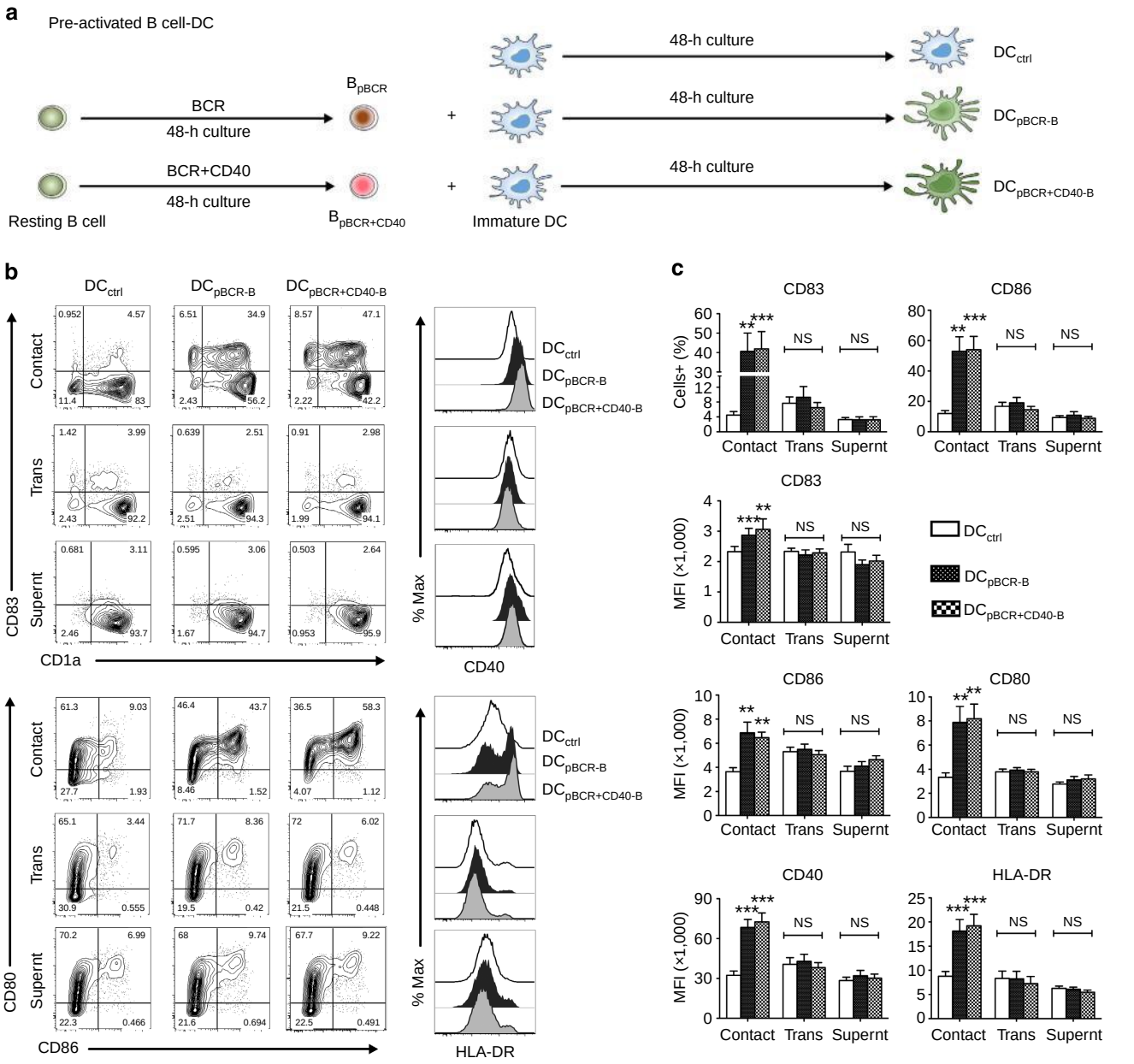

Figure 2 | Cellular contact is required and sufficient for the induction of maturation of DCs by activated B cells. (**a**) Pre-activated B cell-DC experimental design. Immature DCs were cultured in medium containing granulocyte macrophage colony-stimulating factor and IL-4 for 48 h either alone (DC_{ctrl}) or co-cultured at 1:1 ratio with $CD19^+$ B cells that were pre-activated by BCR ($DC_{pBCR\text{-}B}$) or BCR + CD40 ($DC_{pBCR+CD40\text{-}B}$) stimulation. (**b**,**c**) DC–B cell co-culture was either done in 96 U-bottomed wells to allow direct contact between DCs and B cells (Contact) or in transwell plate to separate the B cells from DCs (Trans). Immature DCs were also cultured in the supernatants from activated B cells (Supernt). Phenotypic analysis (% positive cells and mean fluorescence intensity, MFI) of DCs that were gated negative for CD20. Representative plot and mean ± s.e.m. of data from 7 to 11 donors. (Contact, $n=11$; Transwell, $n=7$; Supernatant, $n=8$). $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant by one-way analysis of variance test.

Modulation of DC cytokines and chemokines by activated B cells. During the process of maturation, in addition to upregulation of expression of surface markers, DCs also secrete increased amount of various cytokines and chemokines that play a critical role in creating inflammation, polarization of T cells and migration of diverse immune cells. Analysis of supernatants from DC–B cell co-cultures showed that in the presence of resting B cells ($DC_{Rest\text{-}B}$) only marginal changes in the cytokine profile were observed (Fig. 5a). When B cells were activated directly in the DC–B cell co-culture via BCR signalling ($DC_{BCR\text{-}B}$), B cells stimulated DCs to produce significant amount of IL-6, tumour necrosis factor (TNF)-α and IL-10 (Fig. 5a). Similarly, co-culture of DCs in direct contact with B cells that were pre-activated with either BCR alone ($DC_{pBCR\text{-}B}$) or BCR + CD40 ($DC_{pBCR+CD40\text{-}B}$) significantly enhanced the production of above cytokines (Fig. 5b). However, DC co-cultured with pre-activated B cells in transwell culture system did not produce increased amount of cytokines. As the above cytokines in the co-culture could be derived from DCs as well as B cells, we wanted to confirm that increased secretion of cytokines in DC–B cell co-culture was mainly because of DCs. In fact, intracellular staining showed that activated B cells induced significantly increased frequency of IL-6- and TNF-α-producing DCs (Fig. 5c), suggesting that increased cytokine levels in DC–B cell co-culture were mainly contributed from DCs.

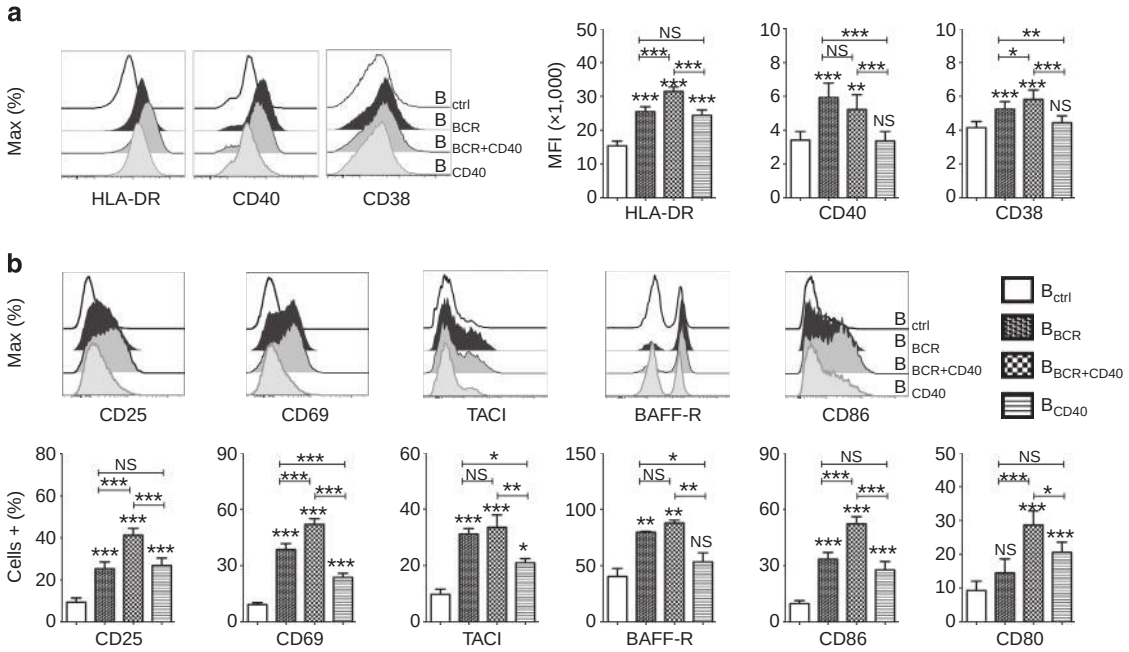

Figure 3 | Phenotypic analysis of B cells activated with various stimuli. CD19$^+$ B cells were either cultured alone (B_{ctrl}) or in the presence of BCR (B_{BCR}) or agonistic CD40 antibody (B_{CD40}) alone, or BCR + CD40 ($B_{BCR+CD40}$) for 48 h. (**a**,**b**) Expression of surface activation markers on B cells as analysed by flow cytometry. Representative plot and mean ± s.e.m. of data from five to ten donors. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS, not significant. Statistical significance as determined by one-way analysis of variance test.

Next, we analysed the supernatant of B cell–DC co-culture for various chemokines that could influence the trafficking of immune cells. Similar to cytokine profiles, irrespective of B-cell activation method, that is, B cells activated directly in the DC:B cell co-cultures via BCR signalling ($DC_{BCR\text{-}B}$) (Fig. 5d) or pre-activated with BCR ($DC_{pBCR\text{-}B}$), or with BCR + CD40 ($DC_{pBCR+CD40\text{-}B}$) before co-culture with DCs (Fig. 5e), stimulated DCs to produce significantly increased amounts of CCL17 and CCL22 (ligands for CCR4), CCL3 and CCL4 (ligands for CCR5), and CXCL8 (IL-8, ligand for CXCR1 and CXCR2). In contrast, CXCL16 (ligand of CXCR6), and CXCL9 and CXCL10 (ligands for CXCR3), were not significantly altered (Fig. 5d,e).

To explore the contribution of DCs to the chemokines in the B cell–DC co-culture, we analysed the supernatants of activated B cells. On BCR (B_{BCR}) or BCR + CD40 ($B_{BCR+CD40}$)-mediated activation, B cells did not secrete CCL17, CCL22 and CXCL16, indicating that these chemokines in DC–B cell co-cultures were contributed mainly by DCs. However, the secretion of CCL3, CCL4, CXCL9 and CXCL10 by activated B cells was either on par with those levels observed in DC–B cell co-cultures or even higher, suggesting that these chemokines in DC–B cell co-cultures were contributed mainly by B cells rather than DCs (Supplementary Fig. 4a). Furthermore, analysis of intracellular expression of chemokines in DCs revealed that activated B cells enhanced the frequency of CXCL8-producing DCs, but not CCL4 (Supplementary Fig. 4b). Together, these findings provide a pointer towards selective induction of DC chemokines by activated B cells.

T-cell stimulation and polarization by B-cell-matured DCs. DCs matured under the influence of B cells were evaluated for T-cell stimulatory capacity by co-culturing B-cell-depleted DCs with CD4$^+$ T cells (Th cells) (Fig. 6a). Corroborating the DC phenotype and cytokine profile, resting B cells did not alter the capacity of DCs ($DC_{Rest\text{-}B}$) to induce T-cell proliferation and was similar to control DCs (DC_{ctrl}). On the contrary, DCs that were 'educated' by activated B cells ($DC_{BCR\text{-}B}$) stimulated significant proliferation of T cells (Fig. 6b) and was associated with significant production of T-cell-derived cytokine IL-2 (Fig. 6c). Further, our results also indicated that DCs that received maturation signals from B cells pre-activated with either BCR alone ($DC_{pBCR\text{-}B}$) or with BCR + CD40-stimulation ($DC_{pBCR+CD40\text{-}B}$) were similar in their ability to stimulate T-cell proliferation and to induce IL-2 in proliferating T cells (Supplementary Fig. 5a). On the contrary, DCs cultured either in transwell plates (Supplementary Fig. 5b) or with supernatants of activated B cells (Supplementary Fig. 5c) did not promote T-cell proliferation and IL-2 secretion, thus further confirming the indispensability of cellular contact between DCs–B cells for the functional maturation of DC.

To further understand the influence of B-cell-matured DCs on CD4$^+$ T-cell responses, we measured the levels of various Th cell cytokines in the DC–T cell supernatants. Interestingly, $DC_{BCR\text{-}B}$ skewed Th-cell responses towards Th2 as indicated by significant secretion of Th2 cell cytokines, including IL-4, IL-5 and IL-13 (Fig. 6d). In addition, modest increase in TNF-α and IL-22 was also observed (Fig. 6e). However, IFN-γ, signature cytokine of Th1 and IL-17, signature cytokine of Th17, and also IL-21 and IL-10 were not altered (Fig. 6f).

B-cell-matured DCs selectively induce Th2 differentiation. Next we investigated the ability of B-cell-matured DCs to modulate the differentiation of naive Th cells. We found that DCs that received signals from BCR-activated B cells ($DC_{BCR\text{-}B}$) significantly increased the frequencies of Ki-67$^+$ and IL-2$^+$ T cells ($T_{BCR\text{-}B\text{-}DC}$) (Fig. 7a,b), indicating enhanced proliferation of naive T cells. However, frequencies of Ki-67$^+$ and IL-2$^+$ T cells were not significantly altered when naive Th cells were co-cultured with DCs that received signals from resting B cells ($T_{Rest\text{-}B\text{-}DC}$). Moreover, naive T cells cultured with $DC_{BCR\text{-}B}$ significantly downregulated the expression of CD27 and CCR7 compared with T cells cultured with DC_{ctrl} or $DC_{Rest\text{-}B}$ (Fig. 7c,d). These results

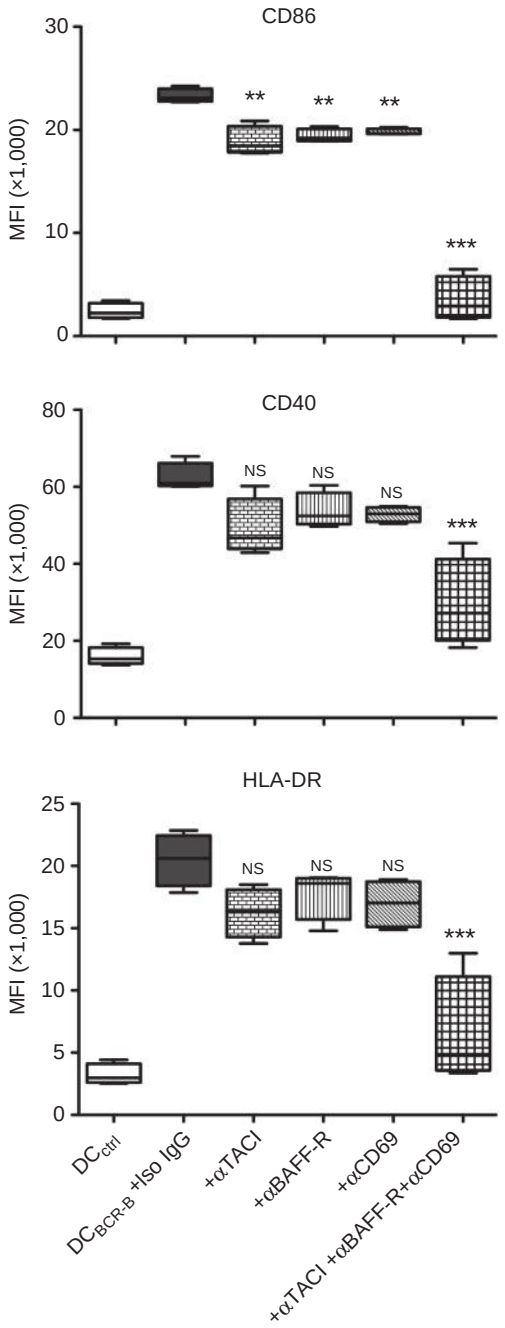

Figure 4 | Several surface molecules on activated B cells are involved in contact-dependent induction of maturation of DCs. Immature DCs were cultured in medium containing granulocyte macrophage colony-stimulating factor and IL-4 for 48 h either alone (DC_{ctrl}), or co-cultured at the ratio of 1:1 with $CD19^+$ B cells in the presence of BCR stimuli. Co-culture was done either in the presence of isotype control mouse IgG (DC_{BCR-B} + Iso IgG) or blocking antibodies to TACI (+αTACI), BAFF-R (+αBAFF-R), CD69 (+αCD69) alone or together (+αTACI +αBAFF-R +αCD69). Flow cytometric analysis of phenotype (mean fluorescence intensity, MFI) of CD20-negative DC population was performed. Mean ± s.e.m. of data from four donors. $^{**}P<0.01$; $^{***}P<0.001$; NS, not significant by one-way analysis of variance test.

suggested that B-cell-matured DCs induce proliferation and differentiation of effector T cells.

Of note, $CCR7^-CD27^-$ T cells were previously shown to contain mainly the IL-4-producing T cells[22]. In line with this report, we further found that DC_{BCR-B} significantly increased the frequency of $CCR4^+$ T cells but did not alter the expression of CXCR3, CXCR5 and CCR6 on T cells (Fig. 7c,d). Of particular relevance, CCR4 has been shown to be preferentially expressed on Th2 cells and Tregs[23]. Interestingly, we found that DC_{BCR-B} significantly increased the frequency of IL-4-expressing cells but not $FoxP3^+$ cells (Fig. 7a,b). Thus, our findings suggest that B-cell-matured DCs selectively induce differentiation of Th cells towards Th2 phenotype and not Tregs. However, the frequency of IFN-γ-expressing Th1 cells and IL-17-secreting Th17 cells was unaltered by DC_{BCR-B} (Fig. 7a,b).

Further, analysis of the supernatants for T-cell cytokines revealed that DC_{BCR-B} significantly increased the secretion of IL-2, which supports the proliferating T cells (Fig. 7e). Interestingly, these T cells ($T_{BCR-B-DC}$) secreted significantly higher amount of Th2 cytokines such as IL-4, IL-5 and IL-13 (Fig. 7e). However, the secretion of IFN-γ and IL-17 by T cells was not significantly altered by DC_{BCR-B} (Fig. 7e). Finally, we examined the expression of Th cell subset-specific transcription factors to substantiate the surface phenotype and cytokine profile data of T cells. DC_{BCR-B} significantly enhanced the expression GATA3, the master regulator of Th2 cells (Fig. 7f)[24], while the expression of T-bet (Th1), RORC (Th17) and Foxp3 (Tregs) was not altered by DC_{BCR-B} (Fig. 7a,b,f). Of note, DCs activated by B cells pre-stimulated with BCR (DC_{pBCR-B}) or CD40 alone ($DC_{pCD40-B}$), or BCR + CD40 ($DC_{pBCR+CD40-B}$), were also capable of inducing naive Th-cell activation, proliferation and differentiation to Th2 phenotype to a similar extent (Supplementary Fig. 6). Taken together, our results demonstrate that DCs that receive signals from activated B cells selectively induce differentiation of Th2 cells from naive T cells.

Th2 polarization by B-cell-matured DCs is OX-40 ligand-dependent. We explored the mechanism of induction of Th2 cells by B-cell-matured DCs. We observed that activated B cells did not induce Th1-triggerring molecules in DCs such as IL-12 and CD70 (Fig. 8a,c), which is in consensus with the unaltered IFN-γ-expressing cells and IFN-γ secretion by T cells that were stimulated by B-cell-matured DCs. Further, B cells did not stimulate the production of IL-33 in DCs, a cytokine that was shown to amplify both Th1- and Th2-type responses in humans (Fig. 8b)[25].

As DCs did not produce Th2-promoting cytokines, we next investigated the expression of Th2-inducing surface molecules on DCs. Interestingly, we found that activated B cells, but not resting B cells, significantly upregulated the expression of OX-40 ligand (OX-40L) on DCs (Fig. 8d). Thus, upregulation of OX-40L in the absence of IL-12 and CD70 expression on B-cell-matured DCs suggested that its interaction with OX-40 on T cells is responsible for driving Th2 polarization. Of particular relevance, epithelial cell-derived thymic stromal lymphopoietin (TSLP) was also shown to upregulate the expression of OX-40L on DCs and is implicated in Th2 responses during allergy reactions[26]. Hence, we compared the phenotype and T-cell-stimulating abilities of TSLP-treated DCs (DC_{TSLP}) and B-cell-matured DCs (DC_{pBCR-B}). Surprisingly, with the exception of CD86, TSLP did not significantly upregulate the activation-associated molecules on DCs and DC_{TSLP} failed to induce significant T-cell proliferation, as compared with DC_{pBCR-B} (Supplementary Fig. 7a,b,d). Interestingly, although the expression of GATA3 was enhanced only by DC_{pBCR-B}, both DC_{TSLP} as well as DC_{pBCR-B} enhanced the production of Th2 cytokines, IL-5 and IL-13 to a similar extent, without modifying IFN-γ secretion (Supplementary Fig. 7e,f). Consistent with this observation, induction of OX-40L expression on DCs by BCR- or CD40-activated B cells was similar to that of TSLP signalling (Supplementary Fig. 7c). These findings further

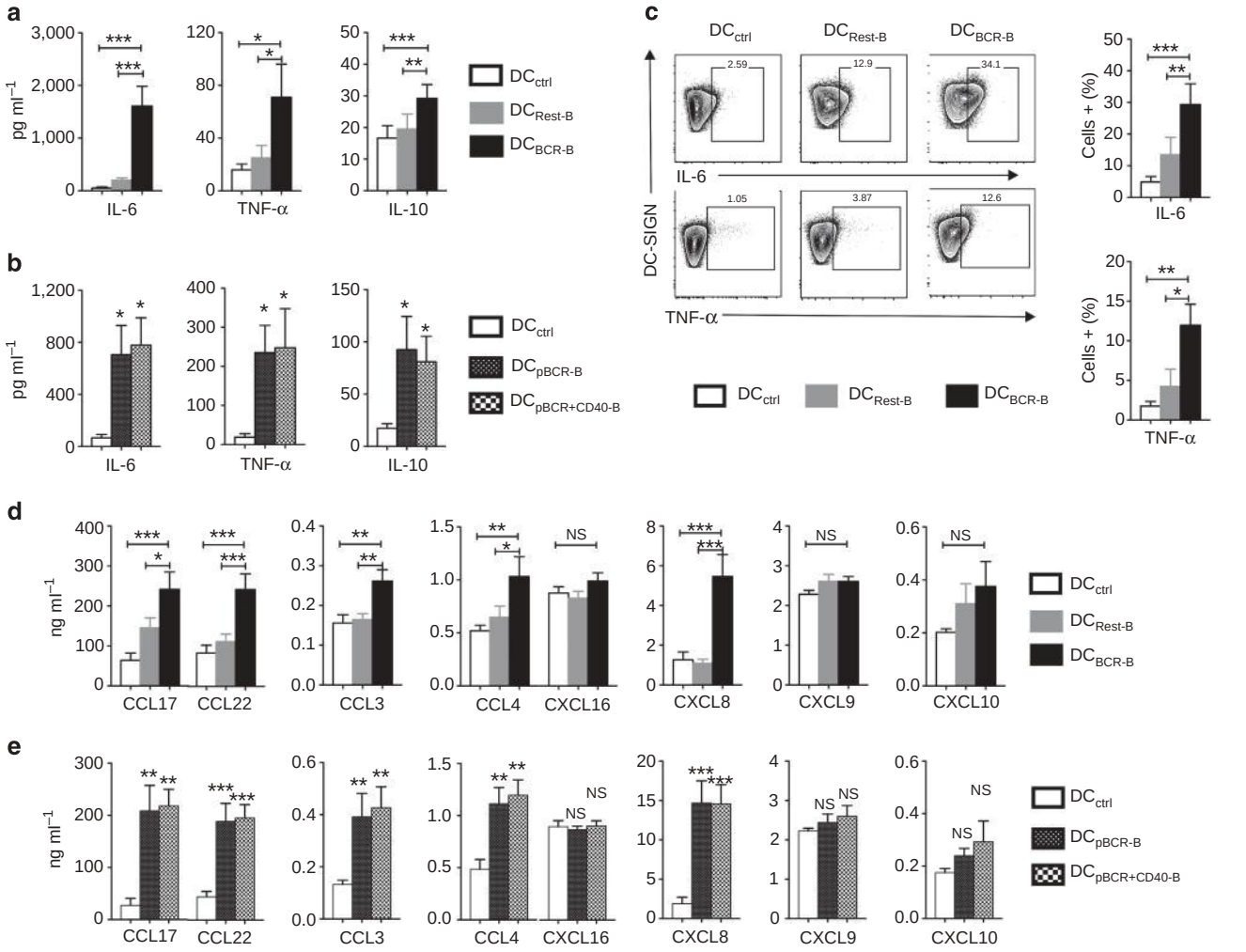

Figure 5 | Effect of activated B cells on the secretion of cytokines and chemokines by DCs. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC_{ctrl}), or co-cultured at 1:1 ratio with $CD19^+$ B cells that were either in resting phase ($DC_{Rest\text{-}B}$) or directly activated in DC-B cell co-culture via BCR stimuli ($DC_{BCR\text{-}B}$). Alternatively, DCs were also co-cultured with $CD19^+$ B cells that were pre-activated by BCR ($DC_{pre\text{-}BCR\text{-}B}$) or BCR + CD40 ($DC_{pre\text{-}BCR+CD40\text{-}B}$) stimulation for 48 h. (**a**,**b**) Supernatants of co-cultures were analysed for the amounts (pg ml^{-1}) of cytokines. Mean ± s.e.m. of data from ten donors. (**c**) CD20-negative DCs from B cell–DC co-cultures were analysed for the intracellular expression of IL-6 and TNF-α by flow cytometry. Representative plot and mean ± s.e.m. of data from four donors. (**d**,**e**) Amount of chemokines in the culture supernatants of DC–B cell co-cultures. Mean ± s.e.m. of data from ten donors. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS, not significant by one-way analysis of variance test.

support that B-cell-matured DC-induced Th2 polarization involves OX-40L.

To confirm this, blocking antibodies to OX-40L were employed in the co-culture of naive T cells and $DC_{BCR\text{-}B}$ (Fig. 8e). Following 6 days of culture, the frequencies of Ki-67^+ and IL-2^+ T cells in the presence of anti-OX-40L antibodies were similar to isotype antibodies (Fig. 8f,g), suggesting that blocking OX-40L did not interfere with DC-induced proliferation and survival of naive T cells. Although blocking of OX-40L did not inhibit the frequency of IL-4-expressing T cells (Fig. 8f,g), there was significant decrease in the amount of secretion of Th2 cytokines, IL-4, IL-5 and IL-13 (Fig. 8h). Together, our results demonstrate that Th2 differentiation by B-cell-matured DCs is dependent at least in part on OX-40 ligand.

Th2 cells induced by B-cell-matured DCs promote IgE production. To further evaluate the physiological role of Th2 polarization induced by B-cell-matured DCs, we co-cultured the differentiated T cells with freshly isolated B cells and determined their ability to induce IgE production (Supplementary Fig. 8a). Preliminary results revealed that Th2 cells induced by B-cell-matured DCs are capable of stimulating B cells to produce IgE, which was similar to that of T cells activated by TSLP–DC (Supplementary Fig. 8b). However, the levels of IgE induced by these Th2 cells were low and variable, and more studies with additional donors are required to provide fully conclusive results.

Discussion

Of much importance in recent advances in B-cell biology, IL-10-secreting regulatory B cells are claimed to restrain the inflammatory responses by inhibiting proinflammatory cytokines, Th1/Th17 cell development and by facilitating Treg differentiation[12,15,27,28]. In addition, B-cell gene therapy approach has been exploited for the induction of antigen-specific immune tolerance[29]. However, pathogenic role of B cells by the way of T-cell stimulation or via autoantibodies is well documented in autoimmune and inflammatory diseases[30–33]. Thus, B cells exert both effector and regulatory functions in co-ordination with

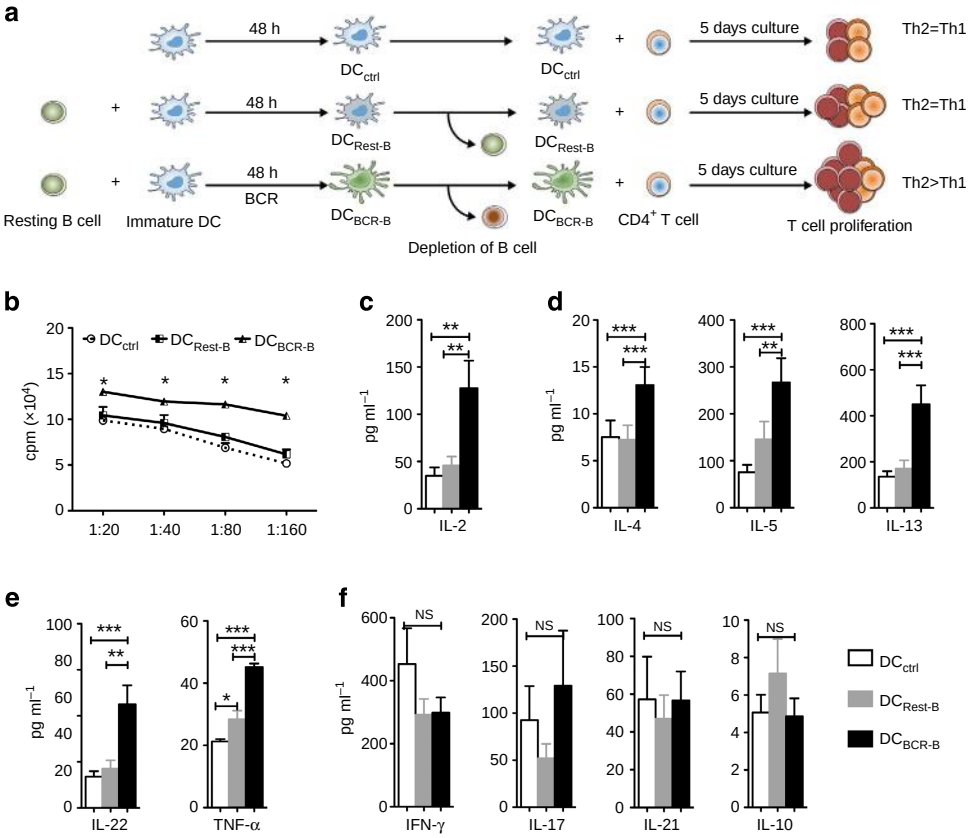

Figure 6 | B-cell-matured DCs induce proliferation of CD4$^+$ T cells producing increased amounts of Th2 type cytokines. (**a**) Experimental design. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC_{ctrl}, or co-cultured at 1:1 ratio with CD19$^+$ B cells that were either in resting phase ($DC_{Rest\text{-}B}$) or directly activated in DC-B cell co-culture via BCR stimuli ($DC_{BCR\text{-}B}$). DCs were purified from B cell–DC co-culture by depleting B cells using CD20 microbeads. CD4$^+$ T cells were co-cultured with DC at various ratios for 4 days. (**b**) DC-mediated CD4$^+$ T-cell proliferation as determined by [^{3}H] incorporation assay. Values are presented as counts per minute (cpm). Data are mean ± s.e.m. from quadruplicate wells and representative of five independent experiments. (**c**–**f**). Supernatants from DC–T cell co-cultures at 1:20 ratio were analysed for different T-cell cytokines. Mean ± s.e.m. of data from eight to ten donors. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS, not significant by one-way analysis of variance test.

T cells and DCs during antigen-specific response. Our results demonstrate that B cells when activated, but not at resting phase, induce maturation of DCs with distinct features to polarize T cells. In consensus with our report, previous studies have also shown that human DC maturation could be induced on interaction with activated immune cells, but not with resting cells, wherein naive and effector T cells, IL-2-activated NK cells, antigen-activated NKT cells and γδ T cells, and also TLR-stimulated neutrophils induce maturation of DCs and increase their T-cell stimulatory capacity[1,3,7–10].

Several reports have demonstrated that B cells have the capacity to regulate the various facets of DC functions both *in vitro* and *in vivo*, to coordinate the process of immune response. B-cell-derived cytokines and chemokines play an important role in the migration of DC subsets. By guiding the expression of CXCL13 on lymphotoxin β-receptor-expressing follicular stromal cells in the spleen, B cell-derived membrane lymphotoxin has been demonstrated to synchronize the migration of a CXCR5$^+$CD11c$^+$ subset of murine DCs for the development of IL-4-producing Th2 cells[34,35]. By binding to CD4, IL-16, a polypeptide cytokine secreted by B cells, could induce migration of circulating human CD4$^+$CD11c$^+$ blood DC subset[36]. As IL-16 could also induce migration of CD4$^+$ T cells, B cells might mediate cross-talk between DC-CD4$^+$T cells–B cells. In addition, via production of antibodies, B cells are also known to regulate DC maturation, activation and functions by engaging Fc receptors or non-Fc receptors, such as CD40, or C-type lectin receptors and T-cell activation[3,37–43].

A previous study in B-cell-deficient mice has indicated that B cells might affect the Th1/Th2 cytokine balance by regulating the function of DCs[18]. The authors found that DCs from B-cell-deficient μMT mice that were injected with keyhole limpet haemocyanin emulsified in complete Freund's adjuvant, induced differentiation of CD4$^+$ T cells that secreted IL-2, IFN-γ, IL-5 and IL-10. However, unlike DCs from wild-type mice, they failed to induce IL-4. Mechanistically, as compared with DCs from wild-type mice, DCs from μMT mice produced higher levels of IL-12 when pulsed with keyhole limpet haemocyanin. This report suggested that B-cell-derived IL-10 might regulate the production of IL-12 by DCs and consequently Th1/Th2 cytokine response. Similarly, DCs from B-cell-deficient mice infected with *Candida albicans* and *Aspergillus fumigatus* also produced higher quantities of IL-12 and lower amounts of IL-10 that might favour enhanced Th1 responses observed in these mice[44]. In line with these reports, B-cell-derived IL-10 was found to be critical for favouring Th2 polarization in *Leishmania* infection model in mice by downregulating IL-12 production by DCs[45]. However, in our study and in another report, CD19$^+$ peripheral human B cells, when activated through BCR alone or with CD40, did not produce significant amounts of IL-10 (ref. 16). These results thus

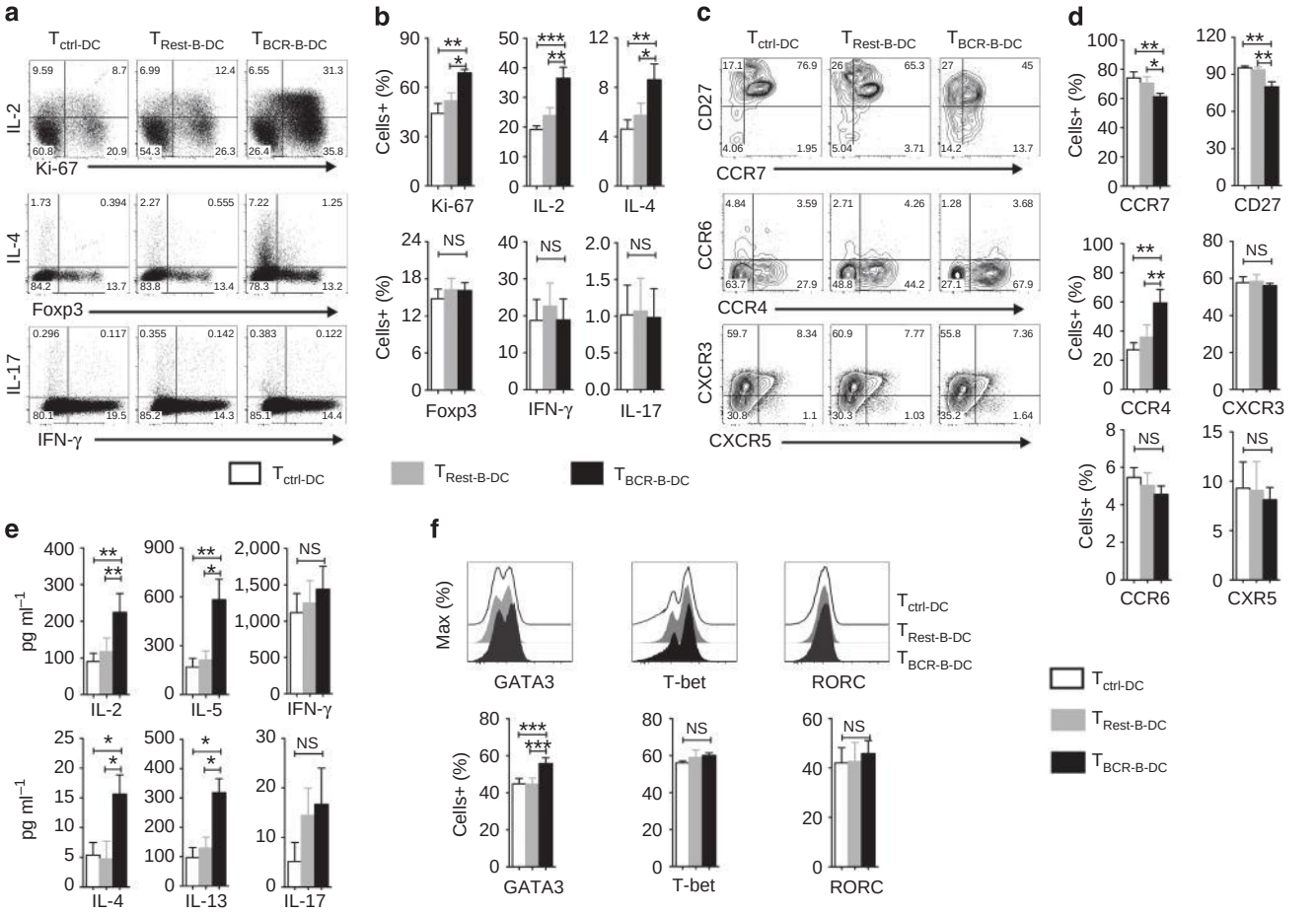

Figure 7 | B cell-matured DCs favour polarization of naive CD4$^+$ T cells to Th2 cells. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC_{ctrl}), or co-cultured at 1:1 ratio with CD19$^+$ B cells that were either in resting phase (DC_{Rest-B}) or directly activated in DC–B cell co-culture via BCR stimuli (DC_{BCR-B}). DCs were purified from B cell–DC co-culture by depleting B cells. CD4$^+$CD45RO$^-$ naive Th cells were co-cultured with control DCs ($T_{ctrl-DC}$) or activated B-cell-matured DCs ($T_{BCR-B-DC}$) or resting B-cell-stimulated DC ($T_{Rest-B-DC}$) at the ratio of 20:1 for 6 days. (**a**,**b**) CD3$^+$ T cells were analysed for the intracellular expression of cytokines and transcription factors by flow cytometry. Representative plot and mean ± s.e.m. of data from ten donors. (**c**,**d**) CD3$^+$ T cells were analysed for the surface phenotype by flow cytometry. Representative plot and mean ± s.e.m. of data from five donors. (**e**) After 6 days, supernatants from DC–T cell co-cultures were analysed for various T-cell cytokines. Mean ± s.e.m. of data from eight to ten donors. (**f**) CD3$^+$ T cells were analysed for intracellular expression of transcription factors by flow cytometry. Representative plot and mean ± s.e.m. of data from four donors. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS, not significant by one-way analysis of variance test.

indicated that B cells could regulate DC cytokines and T-cell polarization both via cytokine-dependent (similar to IL-10) and -independent manner (as described in this report), depending on the context of immune response or possibly depending on the particular subset of B cells.

Morva *et al.*[19] recently reported that B cells that received activation signalling via combination of TLR9 (CpG) and CD40 restrain the maturation and function of human DCs. It is known that TLR9 signalling in combination with BCR stimulation or CD40-mediated activation renders human B cells to gain regulatory properties[12,15,16]. In addition, B cells that receive signals only through TLR9 could also regulate the differentiation and function of DCs[46,47]. All these results suggest that regulatory B cells could suppress the differentiation, maturation and function of DCs, and consequently could play a role in immune tolerance and in preventing inflammatory responses similar to those reported in the case of Tregs. However, our recent results also suggested that the repercussion of DC–B cell cross-talk on DC functions might depend on the type of activation signals received by B cells[20], wherein we found that B cells that received activation signals via BCR alone were not capable of blocking the differentiation of monocyte-derived DCs and did not inhibit TLR-mediated maturation of DCs. These findings are supported by previous reports that have revealed that based on the activation status, B cells might exert either tolerogenic or stimulatory effects on immune cells[48,49].

Here we show that B cells pre-activated with either BCR or CD40 stimulation, or both, are capable of inducing maturation of DCs. Further, we found that contact-dependent interaction mediated by B-cell activation-associated molecules such as CD69, BAFF-R and TACI is involved in DC maturation. Interestingly, several studies have shown that activation-associated molecules of T cells and NKT cells, such as CD69 and CD40L, could modulate maturation and function of APCs, including DCs, monocytes and macrophages[50,51]. CD69 is shown to be involved in the regulation of sphingosine 1-phosphate receptor-1-mediated DC migration into lymph nodes and lymphocyte egress from lymph nodes[52]. Although till date the receptor binding to CD69 is elusive, it could be speculated that B cells on receiving activation signals, upregulate CD69, which

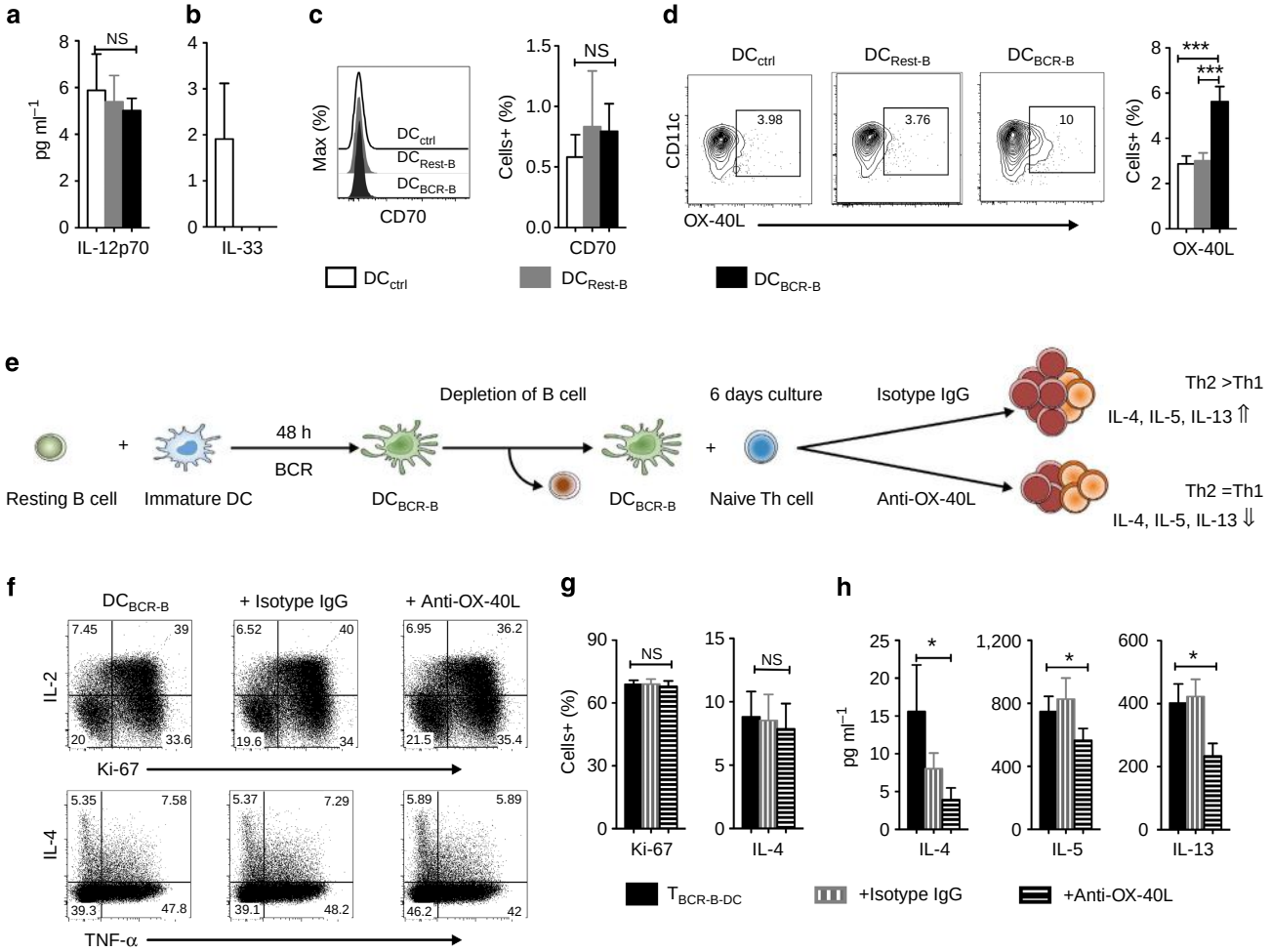

Figure 8 | Th2 polarization by B-cell-matured DCs is dependent in part on OX-40 ligand. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC_{ctrl}), or co-cultured at 1:1 ratio with $CD19^+$ B cells that were either in resting phase (DC_{Rest-B}) or directly activated in DC–B cell co-culture via BCR stimuli (DC_{BCR-B}). (**a**,**b**) Supernatants of DC–B cell co-cultures were analysed for IL-12p70 and IL-33. Mean ± s.e.m. of data from five to ten donors. (**c**,**d**) Expression of CD70 and OX-40L on DCs that were gated negative for CD20. Representative plot and mean ± s.e.m. of data from ten donors. (**e**) Experimental design for OX-40L blocking experiments and repercussion on T-cell cytokines. B-cell-matured DCs were purified from the DC–B cell co-cultures by depleting B cells. $CD4^+CD45RO^-$ naive Th cells were co-cultured with these B-cell-matured DC at the ratio of 20:1 for 6 days ($T_{BCR-B-DC}$) either alone or in the presence of isotype IgG antibody (+Isotype IgG) or blocking antibody to OX-40L +Anti-OX-40 L). (**f**,**g**) $CD3^+$ T cells were analysed for intracellular expression of cytokines or Ki-67 by flow cytometry and (**h**) supernatants from DC–T cell co-cultures were analysed for different T-cell cytokines. Representative plot and mean ± s.e.m. of data from five donors. *$P<0.05$; ***$P<0.001$ NS, not significant by one-way analysis of variance test.

interacts with surface receptor on DCs to induce activation. However, more studies are needed to clarify the role of CD69 in B-cell-mediated regulation of DC maturation and function. Interaction of TACI on B cells with membrane-bound BLys (B-lymphocyte stimulator)/BAFF on DCs was shown to be important for priming naive $CD8^+$ T cells *in vivo*[53], thus supporting our findings on the role of TACI and BAFF-R in DC activation. Notably, despite lower expression of these activation molecules, B cells stimulated with CD40 or CD40L + IL-4 induced DC maturation similar to BCR signalling (Supplementary Fig. 9). Interestingly, although activated naive ($CD27^-CD19^+$) as well as memory ($CD27^+CD19^+$) B cells were capable of inducing DC activation, our preliminary results indicate that naive B cells, but not memory B cells, require both BCR as well as CD40 signalling to be able to induce DC maturation. Based on these findings we believe that in addition to TACI, BAFF-R and CD69, other B-cell surface molecules, yet to be identified, are also required for the induction of DC maturation by activated B cells.

Activated T cells, innate lymphocytes and neutrophils have been shown to induce maturation of DCs with Th1 cell polarization. Further, Th1 polarization was found to be due to enhanced secretion of IL-12p70 by DCs, which is facilitated at least in part by IFN-γ secretion, in case of T and NK cells, and by TNF-α production in case of neutrophils[8–11]. On the other hand, in our study, we found that B-cell-matured DCs did not display significant amount of Th1-inducing molecules such as IL-12p70 and CD70, which explains the unchanged Th1 differentiation. Similarly, lack of specific polarizing cytokines could be the possible reason for B-cell-matured DCs not to favour the differentiation of Th17 cells (IL-1β), follicular Th (Tfh) cells (IL-12 and IL-21) and Tregs (TGF-β). However, B-cell-matured DCs secreted moderate amount of IL-6 and TNF-α, which are the polarizing cytokines for human Th22 (ref. 54). These cytokines could be responsible for the moderate increase in IL-22 expression in T cells by B-cell-matured DCs.

Development of Th2 cells is facilitated by various cytokines, such as IL-4, TSLP and IL-33, in the absence of IFN-γ and IL-12

signalling[26,55,56]. It is well known that DCs do not contribute to Th2 polarization by secreting IL-4. However, T-cell stimulation by DCs in the presence of IL-4 contributed by other immune cells such as basophils, mast cells, eosinophils and naive $CD4^+$ T cells could support Th2 differentiation[55]. DCs could also induce IL-4 production from some of these cells via IL-33. It was demonstrated that IL-33 acts on mast cells via ST2 and activates nuclear factor-κB and mitogen-activated protein kinase[57]. Alternatively, IL-33 could directly initiate the production of Th2 cytokines from polarized Th2 cells *in vitro* and promote IL-4, IL-5 and IL-13 *in vivo*[57]. However, we could not detect IL-33 in the culture supernatant of B-cell-matured DCs, thus ruling out a role for IL-33-mediated Th2 polarization by B-cell-matured DCs.

In addition to cytokines, signalling mediated by interaction of OX-40L, one of the members of the TNF receptor superfamily molecules on DCs, with OX-40 on T cells could also favour Th2 cell responses[58–62]. DCs do not express OX40L constitutively, rather expression of which is induced when cells sense activation signals from antigens. Similarly, naive $CD4^+$ T cells lack the expression of OX-40 and is transiently induced on these cells when they receive activation signals from APCs[63]. We found that along with other co-stimulatory molecules of B7 family, activated B cells significantly upregulated the expression of OX-40L on DCs, which was similar to TSLP–DC. The interaction of these B7 co-stimulatory molecules with CD28 on T cells could sustain the expression of OX-40 on T cells[63]. Of note, blocking OX-40L on DCs significantly inhibited the secretion of Th2 cytokines IL-4, IL-5 and IL-13. Our results thus demonstrate that B-cell-matured DCs could induce Th2 response at least in part through OX-40L. Interestingly, DC-produced IL-6 was also shown to promote Th2 differentiation by inducing STAT3-mediated c-Maf and NFATc2 expression[64–67], and could interfere with development of Th1 cells by upregulating suppressor of cytokine signalling 1 expression[68]. In this context, in addition to OX-40L, IL-6 produced by B-cell-matured DCs might also be involved in the induction of Th2 cells.

We also show that Th2 cells differentiated by B-cell-matured DCs are capable of stimulating IgE production in B cells, similar to TSLP–DC-activated T cells. In fact, previous studies have shown that IL-4 and IL-13 secreted by Th2 cells mediate IgE responses from B cells[69,70]. Therefore, based on these reports, it is likely to be that IL-4 and/or IL-13, secreted by Th2 cells that were differentiated by B-cell-matured DCs, are involved in the production of IgE from B cells. However, further investigations are required to confirm these preliminary results.

In summary, our results indicate that B cells have the ability to control their own effector functions by enhancing Th2-differentiation ability of human DCs. However, promotion of Th2 polarization by B cells via DCs might act as double-edged sword. In case of infection, differentiated Th2 cells might support the production of antibodies from B cells and to neutralize the pathogens and their derived products. Conversely, in autoimmune and allergic diseases, Th2 cells that were polarized by B-cell-matured DCs could amplify autoimmune/inflammatory responses by providing help for autoantibody/IgE-producing B cells. Further, by enhancing the production of chemokines CCL17 and CCL22 in DCs, activated B cells might also promote recruitment of Th2 cells. CCL17 and CCL22 are known to recruit $CCR4^+$ Tregs as well and are expected to act as inflammation-limiting factor in the initial phase of immune response. However, it is well established that Tregs are incompetent to regulate sustained chronic inflammation. Under such inflammatory conditions as in the case of lupus, stimulation of B-cell-derived IL-10 production by CpG-DNA and activation of regulatory B cells could act as inflammation-limiting factor by suppressing the BCR/CD40-activated B-cell-mediated maturation of DCs.

Methods

Reagents and antibodies. The medium used throughout was RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated (56 °C, 30 min) FCS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. $F(ab')_2$ fragments of affinity-purified goat anti-human IgM (Fc fragment-specific) was obtained from Jackson ImmunoResearch Laboratories (West Grove, USA). Cell purification reagents such as microbeads and isolation kits were from Miltenyi Biotec (Paris, France).

Purification of cells. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags of the healthy blood donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (N°12/EFS/079). B cells were isolated from PBMCs by positive selection using the human CD19 microbeads and monocytes were purified by using human CD14 microbeads. Monocytes (0.5×10^6 cells per ml) were cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF; 1,000 IU per 10^6 cells) and IL-4 (500 IU per 10^6 cells) (both cytokines from Miltenyi Biotec) for 5–7 days to obtain immature monocyte-derived DCs.

$CD4^+$ T cells were either selected positively by CD4 microbeads or by negative selection using $CD4^+$ T-cell isolation kit II. Further, from the negatively selected T cells, naive $CD4^+$ T cells were isolated by selectively depleting $CD45RO^+$ T cells by using CD45RO microbeads. The purity of the separated cells was >95% in all the populations.

B-cell stimulation. Freshly isolated B cells were used as 'resting B cells' for co-culture with DCs. For pre-activation, B cells (0.5×10^6 cells per ml) seeded in U-bottom 96-well plates were stimulated via BCR using $F(ab')_2$ of goat anti-human IgM (10 μg ml^{-1}) or with CD40-mediated signalling using agonistic anti-human CD40 monoclonal antibody (mAb; mouse IgG2B clone 82111, R&D Systems, Lille, France; 5 μg ml^{-1}) or with BCR + CD40 or recombinant CD40 ligand (CD40L, R&D Systems; 5 μg ml^{-1}) + IL-4 (500 U ml^{-1}) for 48 h to obtain 'activated B cells'. The agonistic nature of anti-CD40 antibodies was confirmed by various approaches. Anti-CD40 mAbs induced clustering, proliferation and activation of B cells. In a synergistic manner, anti-CD40 agonistic antibodies enhanced B-cell activation and proliferation induced by BCR signalling. Moreover, B cells stimulated with anti-CD40 agonistic antibodies induced significant proliferation of allogeneic T cells.

Supernatants from activated B-cell cultures were collected and centrifuged at 10,000 r.p.m. for 10 min to remove cellular debris. Before co-culture with immature DCs, both resting and pre-activated B cells were treated with mitomycin C (50 μg ml^{-1}; Sigma-Aldrich, Saint-Quentin Fallavier, France) or paraformaldehyde fixed (1% in PBS for 30 min at 4 °C) and washed thoroughly to generate mitotically inactive cells to keep the B-cell number constant in B cell–DC co-culture systems.

B cell–DC co-cultures. Immature monocyte-derived DCs (0.1×10^6 cells per 200 μl per well) with GM-CSF and IL-4 were cultured either alone or with B cells (resting or pre-activated) at the ratio of 1:1 in U-bottom 96-well plates for 48 h. In some experiments, DCs were stimulated with recombinant human TSLP (20 ng per 0.1×10^6 cells, R&D Systems). For transwell experiments, B cells and DCs were kept separated by a 0.4-μm membrane. DCs (0.5×10^6 in 600 μl) were placed in the lower chamber of the transwell plate and pre-activated B cells (0.5×10^6 in 100 μl) in the upper chamber. For the stimulation of DCs using supernatant from activated B cells, immature DCs (0.1×10^6 cells per 250 μl total volume per well) were cultured in GM-CSF and IL-4 alone, or with supernatant from activated B cells (200 μl per well).

In some experiments, resting B cells were directly stimulated in DC–B cell co-culture by using $F(ab')_2$ of goat anti-human IgM (10 μg ml^{-1}). In these experiments, the role of various surface molecules in contact-dependent induction of maturation of DCs by activated B cells was explored by employing blocking mAbs to anti-CD69 (10 μg ml^{-1}), anti-TACI (10 μg ml^{-1}), anti-CD54 (10 μg ml^{-1}), anti-BAFF-R (10 μg ml^{-1}) and anti-CD11a (10 μg ml^{-1}), or mouse IgG isotype control antibodies (all from R&D systems).

DC–T cell co-culture and mixed lymphocyte reaction. Following 48 h of B cell–DC co-culture, B cells were depleted using CD20 microbeads and B-cell-depleted DCs were used for the co-culture with T cells. The purity of DCs following B-cell depletion was up to 98%.

For mixed lymphocyte reaction, graded doses of B-cell-depleted DCs were co-cultured in U-bottom 96-well plate with allogeneic total $CD4^+$ T cells (0.05×10^6 per well per 200 μl) in complete RPMI-1640 medium supplemented with 10% human AB serum. After 4 days of culture, 50 μl of supernatants were collected from each well before pulsing cells with 1 μCi (0.037 MBq) [^{3}H]thymidine overnight to quantify T-cell proliferation. Radioactive incorporation was measured by standard liquid scintillation counting and results were expressed as counts per minute.

For analysing DC-mediated T-cell polarization, DCs were seeded with $CD45RO^-$ naive Th cells (0.1×10^6 per well per 200 μl) in the ratio of 1:20 in U-bottom 96-well plate. For blocking experiments, B-cell-depleted DCs were

pre-incubated with either isotype control antibody or anti-OX40L (10 μg ml^{-1}) for 30 min before co-culture with T cells. Following 6 days, cell-free supernatants were collected to analyse various T-cell cytokines by enzyme-linked immunosorbent assay. Cells were split into two parts and one part of cells was stained for surface molecules and intracellular T-cell transcription factor. The other part was restimulated for intracellular cytokine staining.

Cytokine and chemokine assay. BD Cytokine Bead Array (CBA) kits (BD Biosciences, Paris, France) for human inflammatory cytokines (IL-1β, CXCL8/IL-8, TNF-α, IL-6, IL-10 and IL-12p70) and human Th1/Th2 cytokines (IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ) were used to determine the cytokine levels in the supernatants. IL-33 and IL-17A, and chemokines CCL3, CCL4, CCL17, CCL18, CCL22, CXCL9, CXCL10 and CXCL16 were quantified using DuoSet ELISA kit (R&D Systems). IL-13, IL-17F and IL-22 were analysed using ELISA Ready-SET-Go kits (eBioscience, Paris, France). IL-21 was determined using ELISA MAX Deluxe Sets (BioLegend, San Diego, USA).

Flow cytometry. The following fluorochrome-conjugated antibodies were used at a dilution of 1 in 50. BD Biosciences: fluorescein isothiocyanate-conjugated antibodies to CD1a, CD86, HLA-DR, CD71, IFN-γ and Foxp3; PE-conjugated antibodies to CD25, CD83, CD80, TACI and IL-4; APC-conjugated antibodies to CD19, CD20 and CD40L; Pacific blue-conjugated antibodies to CD4 and CD20; anti-CD69-APC-Cy7; anti-CCR4-PE-Cy7; and anti-CXCR3-AF700. Antibodies from eBioscience included CD40-APC, CD1a-Pacific Blue, CXCR5-PE, IL-6-APC, IL-17A-PE, TACI-PE, BAFF-R-fluorescein isothiocyanate, TNF-α-AF700, IFN-γ-APC-efluor 780, GATA3-PerCP-eFluor 710, T-bet-PE-Cy7, RORC-APC and Foxp3-APC. In addition, antibodies to CD3 (BV605), helios (22F6), CD11c (BV711), CD20 (BV650), CD27 (PE-Cy7), CD80 (BV605), CD206 (APC-Cy7), DC-SIGN (PE-Cy7), CCR7 (BV711), CCR6 (BV605), Ki-67 (BV711), IL-2 (BV650), IL-4 (PE), IL-17A (BV421), OX-40L (PE) and IL-8 (PerCP-Cy5.5) from BioLegend and anti-CD40-PE from Beckman Coulter (Villepinte, France) were also used.

Cells for surface staining were suspended in 10% FCS/PBS and antibodies against surface molecules were added at pre-determined concentration and incubated for 20 min at 4 °C. After washing, cells were fixed with BD Fix buffer and washed before analysis on flow cytometer. The data are presented as percentage of cells positive for indicated molecules or mean fluorescence intensities (MFI) of their expression.

For intracellular staining, DC–T cell co-cultures were stimulated with phorbol myristate acetate (50 ng ml^{-1}; Sigma-Aldrich) and ionomycin (500 ng ml^{-1}; Sigma-Aldrich) at 37 °C for 5 h in the presence of BD golgistop and BD golgiplug during the last 4 h. Following surface staining, cells were fixed and permeabilized (Foxp3 Fixation/Permeabilization kit, eBioscience) according to the manufacturer's instructions, and incubated at room temperature with fluorescence-conjugated mAbs. Cells were acquired on LSRII and processed with FACS DIVA software (BD Biosciences) and analysed by Flowjo.

IgE production by B cells. CD19$^+$ B cells were isolated from PBMC and were cultured in RPMI-1640–10% FCS either alone (0.02×10^6 cells per well per 200 μl) in U-bottomed 96-well plates or with T cells at 1:2.5 ratio for 12 days. Cell-free culture supernatants were collected and quantity of IgE was measured by enzyme-linked immunosorbent assay (Bethyl Laboratories, Euromedex, Soufflweyersheim, France).

Statistical analysis. One-way analysis of variance (repeated measures with Tukey's multiple comparison test) was used to determine the statistical significance of the data. $P < 0.05$ was considered significant.

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Author contributions

M.S.M. and J.B. conceived the study and designed the experiments. M.S.M., M.S., P.H. and E.S.-V. performed experiments. M.S.M., M.S., P.H. E.S.-V., S.V.K. and J.B. analysed the results. B.P provided the advice. M.S.M and J.B wrote the paper.

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Heme oxygenase-1 is dispensable for the anti-inflammatory activity of intravenous immunoglobulin

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Intravenous immunoglobulin G (IVIG) is used in the therapy of various autoimmune and inflammatory conditions. The mechanisms by which IVIG exerts anti-inflammatory effects are not completely understood. IVIG interacts with numerous components of the immune system including dendritic cells, macrophages, T and B cells and modulate their functions. Recent studies have reported that heme oxygenase-1 (HO-1) pathway plays an important role in the regulation of inflammatory response in several pathologies. Several therapeutic agents exert anti-inflammatory effects via induction of HO-1. Therefore, we aimed at exploring if anti-inflammatory effects of IVIG are mediated via HO-1 pathway. Confirming the previous reports, we report that IVIG exerts anti-inflammatory effects on innate cells as shown by the inhibitory effects on IL-6 and nitric oxide production and confers protection in experimental autoimmune encephalomyelitis (EAE) model. However, these effects were not associated with an induction of HO-1 either in innate cells such as monocytes, dendritic cells and macrophages or in the kidneys and liver of IVIG-treated EAE mice. Also, inhibition of endogenous HO-1 did not modify anti-inflammatory effects of IVIG. These results thus indicate that IVIG exerts anti-inflammatory effects independent of HO-1 pathway.

Initially used as replacement therapy in immune deficiencies, IVIG is also widely used for the treatment of a number of autoimmune and systemic inflammatory diseases[1–5]. Despite its therapeutic use for more than three decades, the precise mechanism by which IVIG exerts its beneficial effect is not fully understood. Exploration of mechanisms of IVIG is useful to define the dosage, to identify an appropriate window and duration of treatment, and to delineate biomarkers of therapeutic response. IVIG interacts with numerous components of the immune system including dendritic cells (DCs), macrophages, T and B cells and modulate their functions[6–21]. These mechanisms of IVIG also reflect the functions of circulating IgG in the maintenance of immune homeostasis.

Recent studies in various experimental models such as sepsis, cardiovascular pathologies, experimental autoimmune encephalomyelitis (EAE) and transplantation, and infection models such as *Mycobacterium tuberculosis* have highlighted the biological significance of heme oxygenase-1 (HO-1) enzymatic pathway and the reactive products of this pathway in regulating the inflammation and in the adaptation of the pathogens to the host microenvironment[22–28]. HO-1 catalyzes the degradation of heme, resulting in the liberation of equimolar amounts of iron, carbon monoxide (CO) and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Congenital defects in HO-1 expression in mice and human are associated with systemic inflammation[29]. HO-1 inhibits ovalbumin-induced airway inflammation by enhancing the biological activity of regulatory T cells (Tregs) in an IL-10-dependent manner[30]. Nevertheless, development, maintenance and the functions of Tregs under physiological conditions are not dependent on the activity of HO-1[31].

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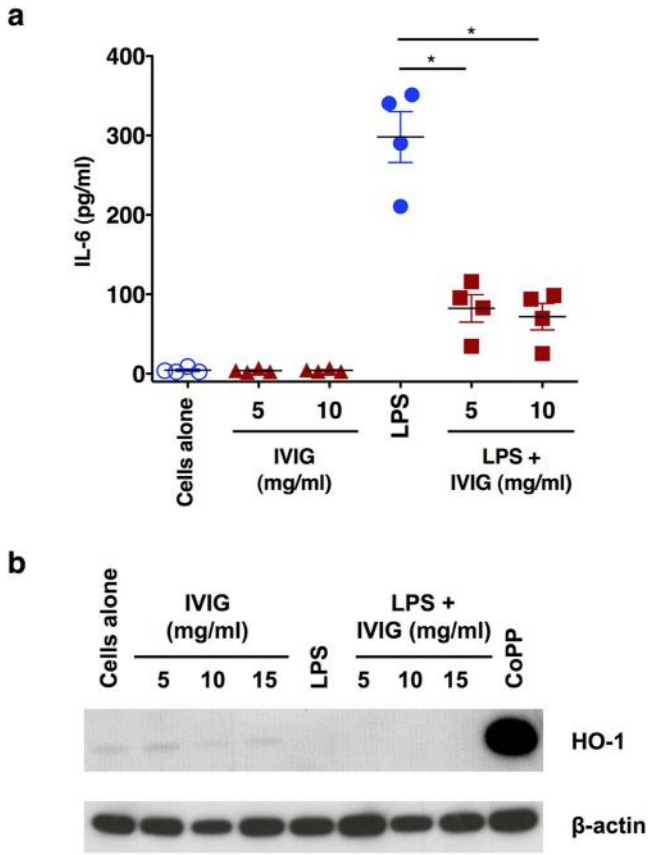

Figure 1. Anti-inflammatory effects of IVIG on human monocytes are not associated with induction of HO-1. (**a**) IVIG suppresses LPS-induced IL-6 production in human monocytes. Human peripheral blood monocytes were cultured in RPMI-1640 medium with 10% fetal calf serum either alone (cells alone) or with IVIG (5 and 10 mg/ml) for 24 hours. In some conditions, after 24 hours of culture, monocytes were exposed to LPS for additional 24 hours. IL-6 in the culture supernatants was measured by ELISA (n = 4). *$p < 0.05$, One-way ANOVA (**b**) Expression of HO-1 in human monocytes treated with IVIG (5, 10 or 15 mg/ml) alone or with LPS during last 24 hours of culture. CoPP was used as a positive control to induce HO-1. Images have been cropped for presentation and full-size blots are presented in Supplementary Figure S2.

CO and biliverdin have potent anti-inflammatory, anti-proliferative, anti-apoptotic, and antioxidant activities and exert their effects on many cell types, including cells of the immune system[32]. CO suppresses the pro-inflammatory response and promotes the anti-inflammatory programs of macrophages, DCs and monocytes[33,34]. Thus, either overexpression of HO-1 in innate cells or exposure to CO leads to inhibition of pro-inflammatory cytokines and enhancement of IL-10. CO also inhibits the lipopolysaccharide (LPS)-mediated maturation of DCs[35,36].

Thus, in view of the common anti-inflammatory role exerted by both HO-1 and IVIG, we investigated if mechanisms of action of IVIG both *in vitro* and *in vivo* implicate HO-1 pathway.

Results

Anti-inflammatory effects of IVIG on human monocytes are not associated with induction of HO-1. It is known that IVIG exerts anti-inflammatory effects on innate cells such as monocytes, DCs and macrophages leading to suppression of inflammatory cytokines[8,37–39]. By analysing the production of IL-6, we first confirmed the anti-inflammatory action of IVIG. Unstimulated monocytes produced insignificant amount of IL-6. However, upon stimulation with LPS, a TLR4-agonist, monocytes produced large amounts of IL-6. Importantly, IVIG significantly reduced the production of IL-6, thus validating the anti-inflammatory effects of IVIG (Fig. 1a). The inhibition however was not dependant on the dose of IVIG.

We then examined the effect of IVIG on the expression of HO-1. Untreated monocytes expressed marginal amount of HO-1 and was not modified by IVIG. Even under inflammatory conditions, IVIG failed to induce the expression of HO-1 (Fig. 1b) in all tested concentrations. The lack of expression of HO-1 in IVIG-treated monocytes was not due to technical errors or non-functioning of HO-1-detecting antibodies as treatment of monocytes with cobalt protoporphyrin IX (CoPP), an inductor of HO-1, strongly induced HO-1. These results thus suggest that anti-inflammatory effects of IVIG on monocytes are independent of HO-1 pathway.

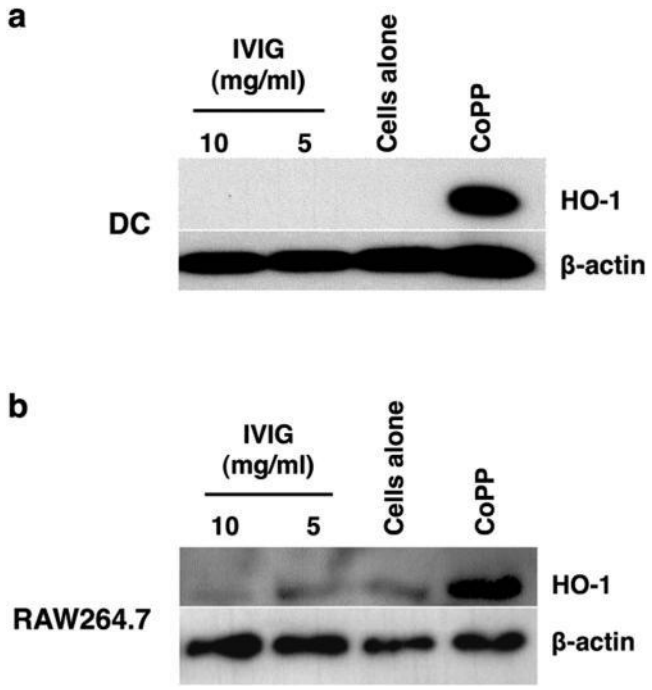

Figure 2. IVIG lacks the capacity to induce HO-1 in dendritic cells and macrophages. (**a**) Human peripheral blood monocyte-derived DCs or **(b)** RAW264.7 macrophages were cultured in the medium alone or with IVIG (5 and 10 mg/ml) for 24 hours. Expression of HO-1 was detected by western blot. CoPP was used as a positive control to induce HO-1. Images have been cropped for presentation.

Inability of IVIG to induce HO-1 in dendritic cells and macrophages. We investigated the effect of IVIG on the expression of HO-1 in other innate cells. Consistent with the results obtained with monocytes, IVIG did not induce HO-1 both in monocyte-derived human DCs as well as RAW264.7 macrophage cell line (Fig. 2a,b). CoPP, the positive control, induced HO-1 in both the cell types. These results thus indicate that inability of IVIG to induce HO-1 is not restricted to particular innate cell.

Inhibition of endogenous HO-1 is not coupled with reduced functioning of IVIG. As innate cells express basal level of HO-1 (Fig. 1b, 2b), we wondered whether inhibition of this endogenous HO-1 is associated with reduced anti-inflammatory action of IVIG. Peritoneal macrophages from C57BL/6J mice and RAW264.7 cells were treated with IVIG for 24 hours and followed by stimulation with LPS. The activation of macrophages by LPS leads to oxidation of L-arginine via nitric oxide synthase and produce nitric oxide (NO). As shown in Fig. 3a,b, IVIG significantly inhibited LPS-induced NO production. However, treatment of cells with tin-mesoporphyrin (SnMP) to suppress enzyme activity of the endogenous HO-1 did not inhibit anti-inflammatory effects of IVIG on NO production (Fig. 3a,b).

The protective effect of IVIG in experimental autoimmune encephalomyelitis is independent of induction of HO-1 *in vivo*. In order to validate the non-involvement of HO-1 pathway in IVIG-mediated anti-inflammatory effects *in vivo*, we resorted to EAE model. EAE was induced in C57BL/6J mice using MOG_{35-55}. Confirming the previous reports, treatment of mice with IVIG significantly delayed the onset of EAE and the severity of the disease (Fig. 4a)[40–42]. However, consistent with *in vitro* results, this protection was not associated with an induction of HO-1 irrespective of the organs examined (liver and kidney) (Fig. 4b). Western blot analysis of lungs and spleen also showed same results. Naive mice injected with CoPP (20 mg/kg) were used as positive control for the expression of HO-1. In fact, expression of HO-1 was confirmed in the liver and kidneys of these mice 24 hours following CoPP injection (Fig. 4b).

Discussion

It was suggested that HO-1 functions as a "therapeutic funnel"[32]. Several reports in experimental models have recently demonstrated that HO-1 pathway and its products could be used for the prevention or treatment of immune-mediated disorders. These protective effects are mediated by multiple functions of HO-1 that include immunosuppression, anti-inflammatory, anti-apoptosis and anti-oxidant effects. The anti-inflammatory mechanisms of HO-1 are mainly via modulation of activation of immune cells including antigen presenting cells and lymphocytes[32]. In fact, IVIG has been demonstrated to modulate the functions of both innate cells and T cells. Thus, IVIG was reported to suppress the activation of DCs, macrophages and monocytes and secretion of inflammatory cytokines while enhancing anti-inflammatory mediators like IL-10 and IL-1ra. In addition, IVIG also inhibits APC-mediated effector T cell activation, proliferation. Recent reports further demonstrate that IVIG inhibits Th1 and Th17 subsets, which are pathogenic in various autoimmune and inflammatory diseases, and

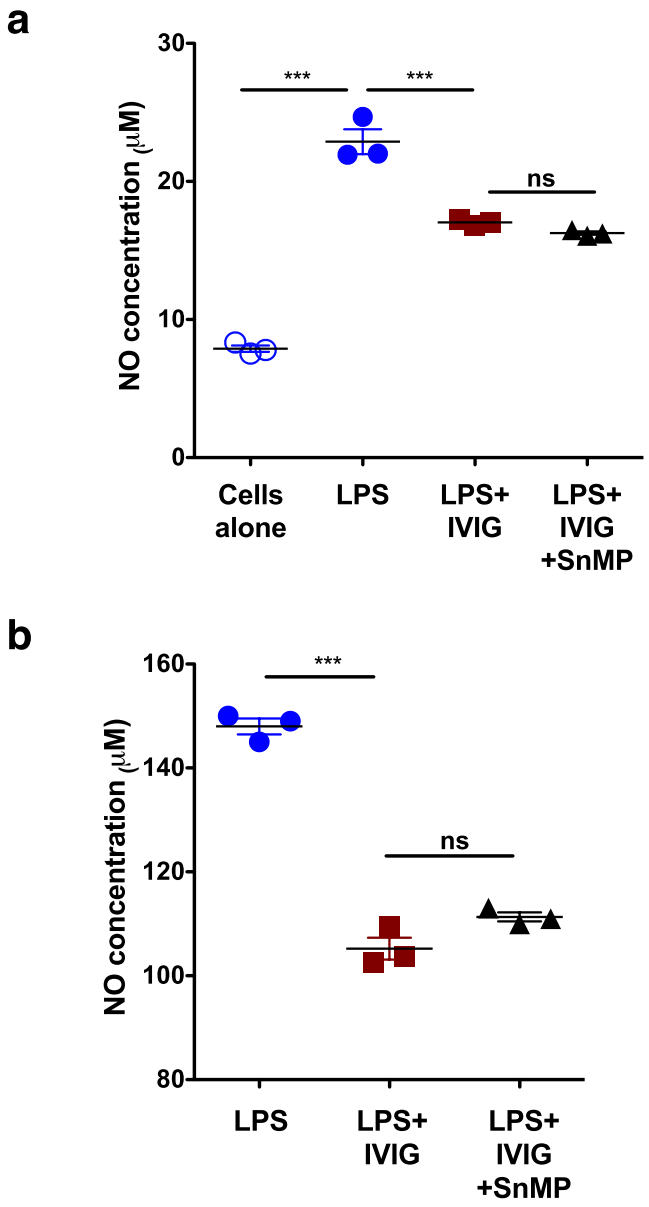

Figure 3. Inhibition of endogenous HO-1 is not associated with loss of anti-inflammatory action of IVIG. (**a**) Peritoneal macrophages from C57Bl/6J mice (n = 3) or (**b**) RAW264.7 macrophages (n = 3) were treated with IVIG (10 mg/ml) for 24 hours. They were then exposed to either LPS alone or LPS and SnMP for additional 24 hours. Production of NO was evaluated by Griess method. ***$p < 0.001$, One-way ANOVA and ns, not significant.

reciprocally expands Tregs[40–48]. Importantly, HO-1 appears to be required for the action of several therapeutic molecules. For example, rapamycin appears not to exert its anti-proliferative effects on smooth muscle cells unless HO-1 is present[49]. All these different lines of evidence underscore the importance of dissection of HO-1 pathway in the anti-inflammatory effects of IVIG.

Previous reports have shown that innate cells express HO-1 in steady state and inhibit toll-like receptor-mediated (such as LPS) activation and secretion of pro-inflammatory cytokines[35,36,50]. Although, the expression of HO-1 in monocytes, DC and RAW264.7 was not prominent, we could detect basal expression of protein by western blot. However, IVIG did not modulate the basal expression of HO-1. Thus, suppression of LPS-mediated IL-6 and NO production by IVIG were independent of HO-1 pathway. It could be argued that IVIG-mediated suppression of IL-6 and NO might be due to passive neutralization of LPS by antibodies as we pre-treated the innate cells with IVIG before stimulation with LPS. However, as analyzed by the expression of CD80 and CD86, IVIG-mediated anti-inflammatory effects was well-preserved in the monocytes even if cells were stimulated with LPS followed by treatment with IVIG (Supplementary Fig. S1), thus ruling out passive neutralization of LPS as a mechanism of anti-inflammatory effect of IVIG. As activation stimuli such as LPS were reported to inhibit the expression of HO-1[35], we opted for examining if IVIG-preconditioning results in HO-1

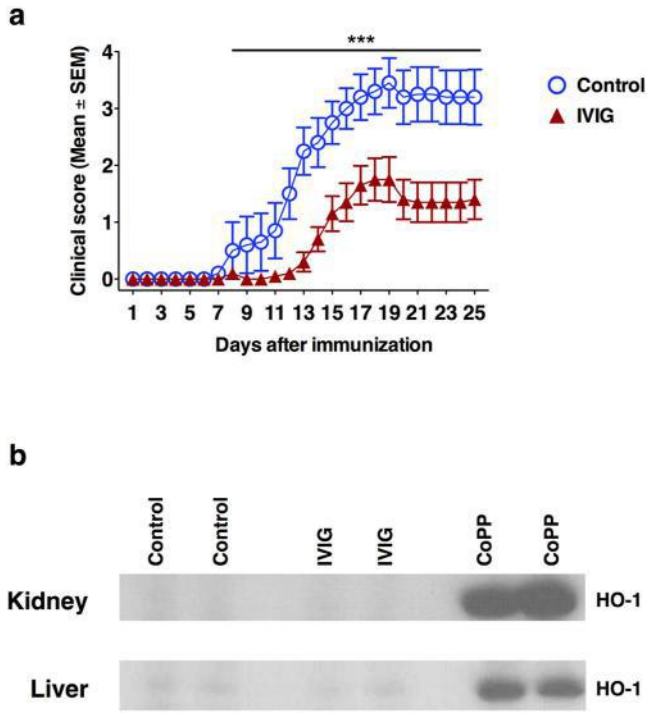

Figure 4. The protective effect of IVIG in EAE is independent of induction of HO-1 *in vivo*. (**a**) Treatment of mice (n = 10) with IVIG significantly delays the onset of EAE and the severity of the disease in C57BL/6J mice. Control mice (n = 10) received 0.2 M glycine while IVIG group received 0.8 g/kg IVIG daily from day 0 to 19. Mean clinical scores (±SEM) are presented. ***$p < 0.001$, two-way ANOVA with Bonferroni post-t-test. (**b**) Expression of HO-1 in kidney and liver of IVIG-treated EAE mice. The mice treated with CoPP were used as a positive control. Images have been cropped for presentation.

expression in innate cells, which in turn inhibits LPS-mediated activation of cells. Our data however clearly demonstrates that IVIG or otherwise, normal circulating antibodies lack the capacity to induce HO-1 and even upon inhibition of endogenous HO-1, IVIG-mediated anti-inflammatory effects were not altered.

Tregs play an important role in the prevention of autoimmune and inflammatory responses[51]. Initial reports have indicated that HO-1 in Tregs is critical for their immune suppression functions[52]. However, subsequent reports have contradicted this data and showed that HO-1 expression in Tregs is not key for immunoregulatory functions of these cells both in human and mice[31,53]. As stimuli from DCs are crucial in Treg expansion, subsequent report showed that HO-1 expression in DCs mediated suppressive functions of Tregs[54]. Despite induction of DC-mediated Treg expansion by IVIG as reported recently by us and others, we could not observe induction of HO-1 in DCs by IVIG suggesting that IVIG targets different pathways for the expansion of Tregs. In fact, several lines of evidence suggest that IVIG could expand Tregs via numerous mechanisms, which might act either independent or inter-linked [18,19,40,42,48]. The modulation of DC functions upon recognition of IVIG via DC-SIGN or DICER constitutes a major event[40,48]. This interaction leads to expression of COX-2-dependent PGE2 production in DCs, which in turn expands Tregs. The role of PGE2 in IVIG-mediated Treg expansion was documented both *in vivo* in animal models and in autoimmune patients treated with IVIG[40,45].

Experimental models have shown that HO-1 pathway inhibits pathogenic T cell responses. EAE has been used as an experimental model for multiple sclerosis and that induction of HO-1 pathway suppress neuro-inflammation in EAE[23]. Induction of HO-1 also suppressed IFN-γ and TNF-α responses of CNS-infiltrating T cells. Suppression of Th1 responses by HO-1 was also reported in type 1 diabetes model in NOD mice[25]. Although modulation of Th17 responses by HO-1 in EAE was not analyzed in the previous report, it is likely that HO-1 suppresses Th17 responses as anti-inflammatory functions of HO-1 in non-eosinophilic asthma were associated with inhibition of Th17 responses[55].

Several therapeutic strategies including injection of tolerogenic cells, recombinant proteins, monoclonal antibodies to inflammatory cytokines, pharmacological agents and oral tolerance have been explored in EAE[56,57]. However, long-term safety issues and particularly in pregnant and lactating women are of major concern with currently used therapies for MS[58]. Promising clinical results in relapsing-remitting multiple sclerosis prompted dissection of cellular and molecular mechanisms of action of IVIG in EAE[58]. We have recently reported that IVIG inhibits both Th1 and Th17 responses in EAE model[41] and similar to HO-1 induction model of Chora *et al.*[23], significantly inhibited CNS infiltration of T cells. Confirming the *in vitro* results, the protection rendered by IVIG in EAE was not associated with HO-1 induction. It was however not surprising given that endogenous expression of HO-1 had no consequence on EAE and daily injection of CoPP was required to induce HO-1 in mice and to protect from EAE[23]. Current data show that without activating HO-1, therapeutic benefits could be obtained in EAE. Thus, our results exemplify the multi-faceted mechanisms of IVIG to exert anti-inflammatory

effects independent of HO-1 pathway. These results could be further consolidated by using HO-1-deficient mice. However, data from such mice would be difficult to interpret due to complex interactions of the HO-1 pathway products with various immune and non-immune cells.

IVIG products are not uniform and display variations with respect to formulation, stabilizing agents and source of plasma of healthy donors. Although these variations could influence the outcome of therapy of primary immunodeficiency patients, all IVIG products appear to have similar therapeutic benefits in autoimmune patients. Of note, when different IVIG preparations were examined for their effect on DC activation, endothelial functions, and Th17 differentiation and expansion, all tested IVIG preparations exerted similar effects[37,59]. In this report, we found that lack of induction of HO-1 by IVIG is not restricted to particular innate cell or IVIG preparation. In addition to Gamunex®, other three IVIG preparations (Sandoglobulin®, Tegeline® and Gammagard®) were also inefficient to induce HO-1. Thus, our data indicate that inability of IVIG to induce HO-1 is a universal phenomenon irrespective of preparations.

Methods

Human Cell culture. Buffy coats from the healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang (EFS), Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee permission N°12/EFS/079) and experiments were performed in accordance with the approved guidelines of INSERM. Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coats by density gradient centrifugation using Ficoll-paque PREMIUM (GE healthcare, France). $CD14^+$ monocytes were isolated from PBMCs by positive selection with CD14 microbeads (Miltenyi Biotec, France). They were cultured for 6 days in RPMI-1640 medium plus 10% fetal calf serum containing GM-CSF (1000 IU/million cells) and IL-4 (500 IU/million cells) to obtain monocyte-derived DCs[60].

Murine macrophages. All animal studies were approved and performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (Université Pierre et Marie Curie Paris) at the pathogen-free animal facility of Centre de Researche des Cordeliers, Paris. Murine peritoneal macrophages were extracted from C57BL/6J mice (purchased from Janvier Laboratories, France) by intraperitoneal lavage and cultured in Dubelcco's Modified Eagle's Medium supplemented with 1% penicillin, 1% streptomycin, 5% amino acids and 5% fetal bovine serum.

Murine RAW264.7 macrophages were maintained in Dubelcco's Modified Eagle's Medium supplemented with 1% penicillin, 1% streptomycin, 5% amino acids and 5% fetal bovine serum.

All cells were maintained at 37 °C in humidified air containing 5% CO_2.

Preparations of IVIG. Gamunex® (Grifols Bioscience, USA) was used throughout the study. In addition, Gammagard®, Sandoglobulin®, and Tegeline® were also used for *in vitro* experiments. They were dialysed against a large volume of PBS followed by RPMI-1640 medium at 4 °C for 18 hours to remove the stabilizing agents. IVIG was used at concentrations of 5, 10 or 15 mg/ml/0.5 million cells.

Animals and EAE. All animal studies were approved and performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (Université Pierre et Marie Curie Paris) at the pathogen-free animal facility of Centre de Researche des Cordeliers, Paris.

Ten-week old C57BL/6J mice (purchased from Janvier Laboratories, France) were injected intraperitoneal with CoPP 20 mg/kg. After 24 hours, liver and kidney were recovered and snap-frozen for western-blot analysis to check the HO-1 expression.

To induce EAE, C57BL/6J mice (10/group) were immunized with 200 μg MOG_{35-55} peptide emulsified in complete Freund's adjuvant (1:1 by volume containing 800 μg of nonviable desiccated *Mycobacterium tuberculosis* H37Rv. In addition, 300 ng of pertussis toxin was given intravenously on the same day and 2 days later. Clinical signs of EAE were assessed daily based on the following scoring system: 0, no signs; 1, tail paresis; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; and 5, moribund. From the day of the immunization until the peak of the disease (day 19), mice received daily intraperitoneal injections of 16 mg (0.8 g/kg) IVIG (Gamunex®). The control groups received equal volumes of 0.2 M glycine, the excipient of Gamunex®).

Detection of HO-1 by Western blot. Human monocytes, DCs and RAW264.7 macrophages (0.5 million cells per ml) were treated with different IVIG preparations and with CoPP 25 μM, the activator of HO-1. After 24 hours, supernatants were removed and cells were lysed with a lysis buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, 10% glycerol and 10 mM Na Fluoride, pH = 6.8). In another set of experiments, following 24 hours treatment of monocytes with IVIG, LPS (10 ng/ml; Sigma-Aldrich, France) was added to the cells to stimulate the monocytes and to induce inflammatory cytokines. After 24 hours, supernatants were removed and cells were lysed.

Liver and kidneys from EAE mice at the peak of the disease (day 19 following induction of EAE) or from the mice injected with CoPP were lysed with the lysis buffer.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinylidene fluoride membrane. HO-1 was detected using an anti-HO-1 rat monoclonal IgG (R&D Systems, France), a horseradish peroxidase-conjugated rabbit anti-rat IgG, and the enhanced chemiluminescence kit. β-actin was detected with a mouse anti-β-actin antibody (Sigma-Aldrich).

Phenotype analysis of monocytes treated with IVIG. Peripheral blood monocytes from the healthy donors were stimulated with LPS for 30 min. They were then exposed to IVIG for 48 hours. The expression of

CD80 and CD86 was analyzed by flow cytometry using PE-conjugated MAbs to CD80 and FITC-conjugated MAbs to CD86 (both from BD Biosciencies).

Cytokine analysis. IL-6 in the cell-free culture supernatants was quantified by ELISA (Ready-SET-Go, eBioscience, France). The detection limit was 2 pg/ml.

Measurement of NO production. Peritoneal macrophages from C57Bl/6J mice and RAW264.7 cells were treated with IVIG (10 mg/ml) for 24 hours. They were then exposed to either LPS alone or LPS and SnMP (25 μM; Frontier Scientific, USA) for additional 24 hours. Production of NO was evaluated by Griess method.

Statistical analysis. Two-way analysis of variance (ANOVA) with Bonferroni's post-test was used to analyze daily clinical score. One-way ANOVA was used to determine the statistical significance of the *in vitro* data. *P* value of less than 0.05 was considered significant.

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Author Contributions

C.G., P.H., M.D., E.S.-V., F.C., M.M. and V.K.S. performed the experiments, C.G., P.H., J.D.D., S.V.K. and J.B. analyzed the data, C.G. and J.B. wrote the paper and all authors reviewed and approved the manuscript.

Additional Information

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Basophils are inept at promoting human Th17 responses

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ABSTRACT

Basophils are the rare granulocytes and play an important role in the polarization of Th2 responses and protection against helminth parasites. In addition, basophils contribute to the pathogenesis of several diseases such as asthma, chronic allergy and lupus. Notably, Th17 cells are also implicated in the pathogenesis of these diseases suggesting that basophils support the activation and expansion of this subset of $CD4^+$ T cells. Therefore, we explored whether basophils promote the expansion of human Th17 cells. We show that basophils lack the capacity to expand Th17 cells and to induce the secretion of Th17 cytokines either directly or indirectly via antigen presenting cells such as monocytes. As human basophils lack HLA-DR and co-stimulatory molecules, their inability to confer T cell receptor- and co-stimulatory molecule-mediated signals to $CD4^+$ T cells might explain the lack of Th17 responses when memory $CD4^+$ T cells were co-cultured with basophils.

1. Introduction

Basophils are the rare granulocytes and represent less than 1% of circulating leukocytes. They play an important role in the polarization of Th2 responses and in the protection against helminth parasites [1–5]. Recent studies have identified several surface markers of human and mouse basophils that could be used for the identification and isolation of these cells. These markers include CD49b (DX5), CD123 (IL-3 receptor α chain), CD200R3 (a disulfide-linked dimeric CD200R-like receptor belonging to the immunoglobulin superfamily), CD203c, 2B4 (or CD244, a 66-kDa protein from the CD2 family), CCR2, CCR3, CD45R (intermediate level of expression) and FcεRI. Further, in contrary to mast cells, basophils are c-Kit^- ($CD117^-$) and this marker could be used to discriminate basophils from mast cells in the tissues [2].

Since long time, basophils have been neglected in immunology due to their low number in the circulation and their shared features with tissue-resident mast cells. However, recent studies indicate that basophils have a major impact on the immune responses and diverse roles of these cells in autoimmune and inflammatory diseases are emerging. Because basophils express several sensing molecules including FcεRI, toll-like receptors (TLRs such as TLR2 and TLR4) and receptors for various cytokines including IL-3, IL-33 and IL-25, basophils can readily respond to various stimuli and release immune modulators such as cytokines, chemokines, histamine and lipid mediators [2]. Therefore, a higher number of activated basophils could tilt the homeostatic balance of the immune system leading to inflammatory conditions.

Activated basophils act as accessory cells to provide Th2 environment and enhance dendritic cell-mediated Th2 responses. In fact, recent reports indicate that the function of basophils in the polarization of Th2 responses is not only important for the protection against helminth parasites but it can also contribute to the pathogenesis of asthma, allergy and autoimmune diseases such as systemic lupus erythematosus [1,2,6–8].

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A newly identified subset of $CD4^+$ T cells namely Th17 cells are also implicated in the pathogenesis of asthma, chronic allergy and lupus suggesting that basophils might support the activation and expansion of this subset of $CD4^+$ T cells [9,10]. Th17 cells express lineage specific transcription factor RORC and IL-17A is the prototype cytokine of these cells. In addition, Th17 cells secrete other inflammatory mediators such as IL-17F and IL-22 [9]. As basophils have an important role in the regulation of immune responses such as T and B cell responses, we explored whether basophils promote the expansion of human Th17 cells.

2. Materials and methods

2.1. Isolation of circulating human basophils and monocytes

Buffy coats of healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France upon ethical committee permission (N°12/EFS/079). Basophils from the buffy coats were isolated by two-step process. By percoll density gradient centrifugation, we first obtained peripheral blood mononuclear cells (PBMCs). These PBMCs were subjected to MicroBead-based negative isolation of basophils by using basophil isolation kit II (Miltenyi Biotec, Paris, France) [11]. Monocytes from PBMCs were purified by using CD14 MicroBeads (Miltenyi Biotec). The purity of basophils as well as that of monocytes was in the range of 94 ± 5% as analyzed by flow cytometry (BD LSR II, BD Biosciences, Le Pont de Claix, France). Basophils were analyzed by using fluorochrome-conjugated mAbs to CD203c (eBioscience, Paris, France) FcεRI and CD123 (both from Miltenyi Biotec) [12] while monocytes were monitored by using fluorochrome-conjugated mAb to CD14 (BD Biosciences).

2.2. Isolation of memory $CD4^+$ T cells

To isolate memory $CD4^+$ T cells, untouched total $CD4^+$ T cells were first purified from PBMCs by using $CD4^+$ T-cell isolation kit II (Miltenyi Biotec). Further, by using CD45RA MicroBeads (Miltenyi Biotec), naïve $CD4^+CD45RA^+$ T cells were depleted from total $CD4^+$ T cells. Finally, $CD4^+CD45RO^+CD25^-$ memory T cells were obtained by depleting $CD25^+$ cells with CD25 MicroBeads (Miltenyi Biotec). The purity of isolated cells was in the range of 95 ± 4%.

2.3. Co-culture of basophils and monocytes with $CD4^+CD45RO^+CD25^-$ memory T cells

Allogeneic memory $CD4^+$ T cells were cultured in U-bottomed 96 wells plate (0.1×10^6 cells/200 µl/well) in X-vivo-10% human AB serum and IL-2 (100 IU/0.5×10^6 cells, ImmunoTools, Friesoythe, Germany) either alone; or with basophils in the presence of IL-3 (100 ng/1×10^6 cells, Miltenyi Biotec) or IL-3 and monoclonal anti-human IgE (10 ng/0.1×10^6 cells, clone GE1, Sigma–Aldrich, Saint Quentin Fallavier, France); or with peptidoglycan-stimulated monocytes (5 µg/0.5×10^6 cells, Invivogen, Toulouse, France); or with peptidoglycan-stimulated monocytes and IL-3-primed basophils; or with peptidoglycan-stimulated monocytes and IL-3-anti-IgE-treated basophils. The activation of basophils was analyzed by the expression of CD63 by using fluorescence-conjugated mAb (BD Bioscience). Monocytes and basophils were stimulated in the co-culture and were not pre-activated. The ratio of memory $CD4^+$ T cells and monocytes and/or basophils was maintained at 5:1. After 5 days of culture, the cells were harvested and cell-free culture supernatants were collected for the analysis of IL-17A and IL-17F. The cells were processed for staining and flow cytometry as described below.

2.4. Intracellular staining and flow cytometry

The harvested cells were re-stimulated with phorbol 12-myristate 13-acetate/ionomycin (Sigma–Aldrich) for 6 h, with GolgiStop (BD Biosciences) during last 3 h. Surface staining was done with fluorescence-conjugated CD4 mAb (BD Biosciences) and fixable viability dye (eBioscience), in order to gate and analyze viable $CD4^+$ T cells. Further, cells were fixed, permeabilized (Fix/Perm; eBioscience), and incubated at 4 °C with fluorochrome-conjugated mAbs to IFN-γ, IL-4 (BD Biosciences) and IL-17A (eBioscience). The stained cells were subjected to flow cytometry (BD LSR II). Ten thousand cells were acquired for each sample and data were processed by using FACS DIVA software (BD Biosciences).

2.5. Cytokines analysis

Levels of IL-17A (DuoSet ELISA kits, R&D Systems), IL-17F and IL-6 (ELISA Ready-SET-Go, eBioscience) in cell-free culture supernatants were quantified by ELISA. The detection limits were 15 pg/mL for IL-17A, 16 pg/mL for IL-17F and 2 pg/mL for IL-6.

2.6. Measurement of plasma IgE

The IgE in the plasma of healthy donors was measured by an automated classical sandwich immunoassay by ImmunoCap technology (Thermo Fischer, Phadia SAS, St. Quentin Yvelines, France). Results are expressed in kU/L and the admitted correspondence is 2.4 ng/mL per kU/L.

2.7. Statistical analysis

Statistical analysis was done by one-way ANOVA (Friedman test) or two-tailed Student's-*t*-test using Prism 5 software (GraphPad softwares). Values of $P < 0.05$ were considered as statistically correlated.

3. Results

3.1. Activated human basophils lack the capacity to promote Th17 expansion

We investigated the direct effect of basophils on the expansion of Th17 cells. As stimulated basophils are known to secrete variety of cytokines and other chemical mediators, we also examined if enhanced degranulation of basophils through FcεRI cross-linking would augment Th17 responses. IL-3-primed basophils were co-cultured with $CD4^+CD45RO^+$ memory T cells either in the presence or absence of FcεRI cross-linking. To avoid nonspecific stimulatory effects of xeno-proteins in the fetal calf serum, we utilized X-vivo medium-containing 10% human AB serum for the experiments. Also, survival of basophils in the co-cultures was ensured by the addition of IL-3 at the time of co-culture of cells. As activated $CD4^+$ T cells produce IL-3, this will further ensure the survival of basophils [13,14].

FcεRI cross-linking led to activation of basophils as analyzed by the expression of CD63 (Fig. 1A). We observed that neither IL-3-primed nor FcεRI-activated basophils could amplify $IL\text{-}17A^+$ Th17 cells from memory $CD4^+$ T cells (Fig. 1B and C). The percentage of $IL\text{-}17A^+/IFN\text{-}\gamma^-$ and $IL\text{-}17A^+/IFN\text{-}\gamma^+$ T cells remain unaltered in the presence of either IL-3-primed or FcεRI-activated basophils. In addition, basophils did not activate Th17 cells to secrete Th-17-derived cytokines. Only marginal changes in the secretion pattern of IL-17A and IL-17F were observed (Fig. 2A and B). Thus, our results imply that basophils alone are poor inducers of Th17 cell expansion and hence ruled out the possibility of the direct

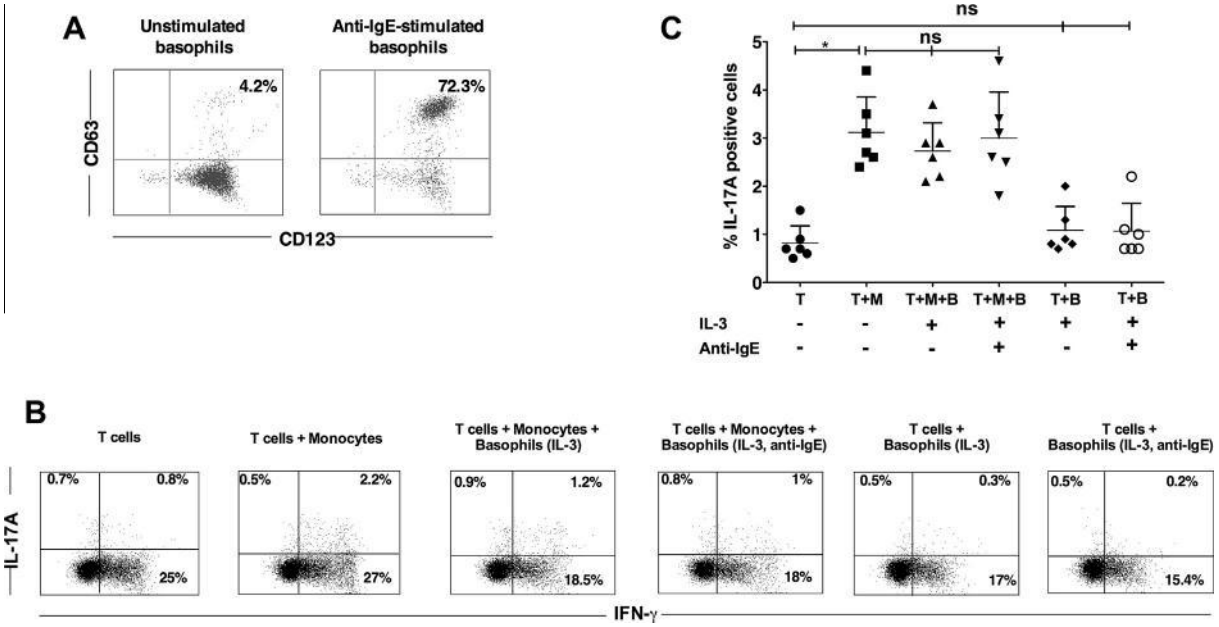

Fig. 1. Human basophils are mute-spectators in Th17 expansion. (A) The expression of CD63 on the surface of unstimulated and anti-IgE stimulated basophils. (B and C) Memory $CD4^+$ T cells were cultured alone with IL-2 (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or combination of IL-3 and anti-IgE. (B) A representative flow-cytometry analysis of intracellular IL-17A and IFN-γ, and (C) percentage (mean ± SD) of $CD4^+CD45RO^+$ memory T cells positive for $IL\text{-}17A^+$ ($n = 6$) were shown. $^*P < 0.05$; ns, not-significant as analyzed by one-way ANOVA test.

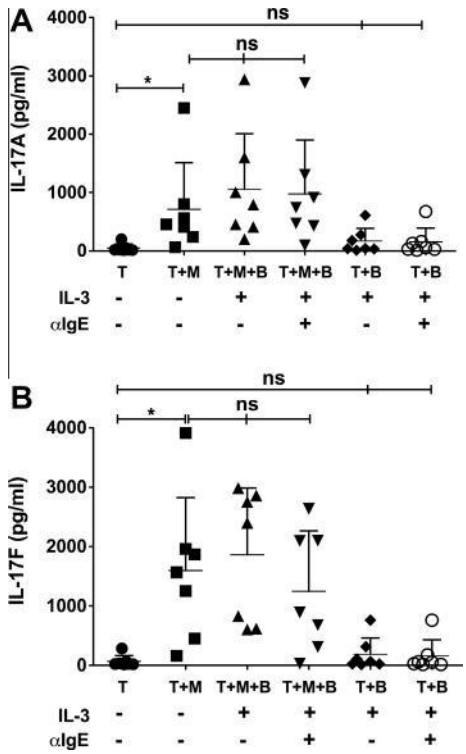

Fig. 2. Human basophils do not promote Th17 cytokine secretion. (A and B) The amount of secretion (pg/mL) of (A) IL-17A and (B) IL-17F in the culture supernatants of memory $CD4^+$ T cells that were cultured alone with IL-2 (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or combination of IL-3 and anti-IgE. The cytokines were measured by ELISA. The data represent mean ± SD from six independent experiments using cells from different donors. $^*P < 0.05$; ns, not-significant as analyzed by one-way ANOVA test.

association of basophils in the development of Th17 responses. We also analyzed the proportion of $IFN\gamma^+CD4^+$ T cells and $IL\text{-}4^+CD4^+$ T cells among $CD4^+$ T cells that were co-cultured with basophils. We observed an increased tendency of Th2 response and decreased Th1 response. However, results were statistically non-significant due to variations among the individual donors (data not shown).

3.2. Activation of basophils is not influenced by the donor-dependent variations in the level of plasma IgE and the expression of FcεRI on the basophils

We examined whether the concentration of IgE in the plasma of healthy donors and the expression of FcεRI on the basophils influence the activation of basophils. We found that donors had uniform level of plasma IgE (28.25 ± 5.1 kU/L, $n = 7$) (Fig. 3A) and the expression of FcεRI on the basophils (mean fluorescence intensity: 6367 ± 1045, $n = 8$) (Fig. 3B). These data thus ruled out the possibility of significant donor-dependent variations in basophil stimulation due to plasma IgE and FcεRI expression on the basophils.

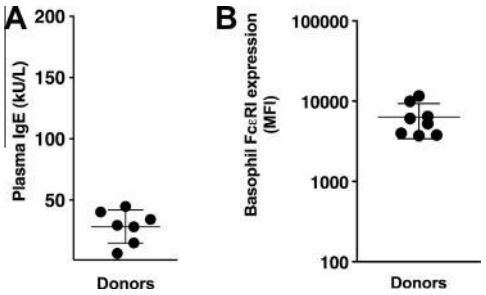

Fig. 3. FcεRI-mediated activation of basophils is not influenced by the level of plasma IgE and the expression of FcεRI on the basophils. (A) The level of IgE (kU/L) in the plasma of healthy donors ($n = 7$). (B) The expression (MFI) of FcεRI on the basophils of healthy donors ($n = 8$). The lines represent mean and SD values.

3.3. Human basophils are inapt at promoting antigen presenting cell-mediated Th17 expansion

It is known that basophils secrete various inflammatory mediators and hence could influence the activation of other immune cells [2,15]. Therefore, by mimicking closely the tissue microenvironment i.e., in the presence of activated antigen presenting cells (APCs, TLR2-activated monocytes in our experiments) that would provide all different signals required for $CD4^+$ T cell activation, we investigated the effect of activated basophils on APC-mediated Th17 responses.

In line with previous reports, we found that IL-$17A^+$ Th17 cells were significantly enhanced when memory $CD4^+$ T cells were co-cultured with monocytes, thus confirming the ability of activated APCs to expand Th17 cells [9,10,16]. Whereas, IL-3 treated basophils did not further amplify monocyte-mediated Th17 responses (Fig. 1B and C). The proportion of IL-$17A^+$/IFN-γ^- and IL-$17A^+$/IFN-γ^+ T cells was not significantly altered in the presence of IL-3-primed basophils with monocytes (Fig. 1B and C). Interestingly, similar results were also obtained in the presence of FcεRI-activated-basophils. These flow-cytometry results were further confirmed by the analysis of secretion of Th-17-derived cytokines. Monocytes significantly enhanced the production of IL-17A and IL-17F by ten to fifteen times (Fig. 2A and B). Although, there was a slight increase in the production of these cytokines in the presence of basophils, the values were not statistically significant (Fig. 2A and B). We have recently demonstrated that basophils also lack the capacity to modulate another Th17 cytokine IL-22 from $CD4^+$ T cells [17]. Together, these results thus provide a pointer that circulating human basophils lack the capacity to enhance APC-mediated Th17 responses.

3.4. Human basophils produce minute amounts of IL-6 following activation

A slender increase in the production of monocyte-mediated Th17 cytokines in the presence of activated basophils suggest that basophils secrete cytokines or soluble factors that stimulate Th17 cytokines. However, human basophils produce undetectable levels of Th17 propagating cytokines such as IL-23 and PGE_2 [18]. On the other hand, basophils have been shown to secrete small amounts of IL-6 that could explain marginal increase in the level of Th17 cytokines. In fact, IL-3 and FcεRI-activated-basophils (0.2×10^5 -cells) produced 57.4 ± 52.8 pg ($n = 4$) of IL-6. However, equivalent number of TLR2-activated monocytes produced 4829.5 ± 1426.3 pg ($n = 4$) of IL-6 (Fig. 4). As activated innate cells such as monocyte, macrophages and dendritic cells (DCs) secrete massive quantities of Th17-amplifying cytokines [19,20], the basophil-secreted IL-6 effect would be nullified.

4. Discussion

Various receptor–ligand interactions between APCs and responder $CD4^+$ T cells, and cytokine milieu in the microenvironment determine the activation, polarization and expansion of $CD4^+$ T cells. Previous reports have shown that murine basophils at secondary lymphoid organs display the features of professional APCs and polarize Th2 responses [21–24]. However, these reports are contradictory due to the basophil depletion method employed [25,26] and also DCs could mediate Th2 polarization independent of IL-4 via Notch ligand Jagged and OX-40 ligand [27,28]. In contrast to murine basophils, several reports including ours demonstrated that circulating human basophils lack HLA-DR and co-stimulatory molecules CD80 and CD86 and were unable to function as APCs to promote T cell polarization [11,29–32]. Although, stimulation of basophils with GM-CSF and IFN-γ was shown to induce HLA-DR expression

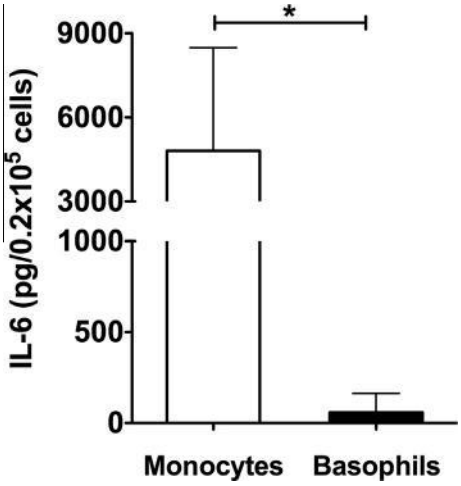

Fig. 4. Human basophils produce minute amounts of IL-6. Basophils were stimulated with a combination IL-3 and anti-IgE for 24 h. Monocytes were activated with peptidoglycan. IL-6 in the culture supernatants was quantified (pg/0.2×10^5 cells) by ELISA. The results are mean ± SD from four donors. $^*P < 0.05$ as analyzed by two-tailed Student's-*t*-test.

to a smaller extent in some donors, these cells did not express co-stimulatory molecules [33]. Thus, the inability of human basophils to confer T-cell receptor- and co-stimulatory molecule-mediated signals to $CD4^+$ T cells might explain the lack of Th17 responses when $CD4^+$ T cells were co-cultured with basophils.

Recently Wakahara et al. demonstrated that human basophils enhance Th17 responses upon interaction with memory $CD4^+$ T cells [34]. The reasons for the discrepancies in the results are not clear. Differences in the type of serum used and stimulatory conditions could be the possible reasons. Based on their results and the presence of basophils in the inflamed mucosal tissues, Wakahara et al. also suggested a role for basophils in the pathogenesis of inflammatory bowel disease [34]. However, on the contrary, a recent report demonstrates that basophils limit the disease severity in experimental murine colitis model [35]. Also, a recent randomized, double-blind placebo-controlled clinical trial failed to demonstrate effectiveness of a human anti-IL-17A monoclonal antibody Secukinumab for moderate to severe Crohn's disease [36]. Therefore, the pathogenic role of Th17 cells in inflammatory bowel disease remains controversial.

To conclude, our results indicate that basophils lack the ability to augment Th17 cell responses either directly or via APCs. Therefore, we suggest that increased activation and accumulation of Th17 cells in various inflammatory diseases such as asthma, chronic allergy and lupus are under the control of innate cells such as monocytes, macrophages or DCs but not basophils.

Author contributions

M.S. performed the experiments, analyzed the data, drawn the figures and wrote the paper.

E.S.-V. performed the experiments and analyzed the data.

P.P. performed the experiments and analyzed the data.

S.V.K. analyzed the data.

J.B. analyzed the data, drawn the figures and wrote the paper.

All authors reviewed the manuscript and approved the final version.

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Intravenous immunoglobulin-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients

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Intravenous immunoglobulin (IVIg) is used in the therapy of various autoimmune and inflammatory diseases. Recent studies in experimental models propose that anti-inflammatory effects of IVIg are mainly mediated by α2,6-sialylated Fc fragments. These reports further suggest that α2,6-sialylated Fc fragments interact with DC-SIGN$^+$ cells to release IL-33 that subsequently expands IL-4-producing basophils. However, translational insights on these observations are lacking. Here we show that IVIg therapy in rheumatic patients leads to significant raise in plasma IL-33. However, IL-33 was not contributed by human DC-SIGN$^+$ dendritic cells and splenocytes. As IL-33 has been shown to expand basophils, we analyzed the proportion of circulating basophils in these patients following IVIg therapy. In contrast to mice data, IVIg therapy led to basophil expansion only in two patients who also showed increased plasma levels of IL-33. Importantly, the fold-changes in IL-33 and basophils were not correlated and we could hardly detect IL-4 in the plasma following IVIg therapy. Thus, our results indicate that IVIg-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients. Hence, IL-33 and basophil-mediated anti-inflammatory mechanism proposed for IVIg might not be pertinent in humans.

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal pooled immunoglobulin G (IgG) obtained from the plasma of several thousand healthy donors. High-dose IVIg (1–2 g/kg) is widely used in the treatment of various autoimmune and inflammatory diseases including Kawasaki disease, idiopathic thrombocytopenic purpura, Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, autoimmune blistering diseases, inflammatory myopathies, graft versus host disease and others[1–4]. The cellular and molecular mechanisms of action of IVIg in these diverse diseases remain incompletely understood. However, available evidence both from experimental and clinical studies provide an indicator that IVIg could benefit these diverse diseases via several mutually non-exclusive mechanisms[2,5–10]. These mechanisms include inhibition of activation and functions of innate immune cells such as dendritic cells (DCs), monocytes, macrophages and neutrophils; inhibition of pathogenic effector T cells such as Th1 and Th17 cells; expansion of regulatory T cells (Tregs); modulation of B cell responses; and inhibition of complement pathways. In addition, IVIg has been shown to inhibit inflammatory cytokines and to augment anti-inflammatory molecules such as IL-10 and IL-1 receptor antagonist[11–21].

IgGs are glycoproteins and contain fragment antigen-binding (Fab) regions that recognize antigens, and fragment crystallizable (Fc) regions that exert effector functions upon binding to Fcγ receptors. The Fc fragments are glycosylated at Asn297 and recent studies in animal models advocate that anti-inflammatory effects of IVIg

are mediated by a small fraction of antibodies that contain terminal α2,6-sialylated glycans at Asn297. It was proposed that α2,6-sialylated Fc fragments interact with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin-positive (DC-SIGN$^+$) innate cells to release IL-33, which subsequently expands IL-4-producing basophils[22]. However, translational insights on these observations are lacking. Therefore, we investigated whether high-dose IVIg therapy induces IL-33 production in autoimmune patients, which in turn would mediate basophil expansion and IL-4 responses.

Results

IVIg therapy induces IL-33 in autoimmune patients. Previous work on the role of IL-33 in IVIg-mediated anti-inflammatory effects was performed in K/BxN serum-induced murine arthritis model. It should be noted that IVIg is not recommended for rheumatoid arthritis due to its inefficacy to relieve inflammation[4]. Therefore, K/BxN serum-induced murine arthritis model might not provide factual image of the mechanisms of IVIg in autoimmune patients. Earlier studies have indicated that IVIg therapy benefits patients with inflammatory myopathies[1,4]. Therefore, by using heparinized blood samples of these patients (cohort 1 patients), we first investigated the repercussion of IVIg therapy on the induction of IL-33. We found that, out of nine patients, six had minimal level of plasma IL-33 prior to IVIg therapy. The pre-IVIg plasma level of IL-33 was in the range of 150.75 ± 79.52 pg/ml (n = 9) (Fig. 1a). Following IVIg therapy, with an exception of one patient, all remaining patients had significant raise in plasma IL-33 and was in the range of 492.23 ± 130.30 pg/ml (n = 9) (Fig. 1a). However, the increase in IL-33 following IVIg therapy was heterogeneous and was varying from 1.2 to 911-fold.

To confirm these results, we analyzed the plasma samples from another cohort of patients with inflammatory myopathies (n = 4) or anti-neutrophil cytoplasmic antibody-associated vasculitis (n = 3) (cohort 2 patients). Importantly, these patients also showed significant increase in plasma IL-33 following IVIg therapy (Fig. 1b) thus confirming the results obtained with cohort 1 patients. The pre-IVIg plasma level of IL-33 was 80.43 ± 24.93 pg/ml (n = 7) that increased to 291.58 ± 34.40 pg/ml following IVIg therapy. Together, these results indicate that irrespective of pathologies, IVIg therapy in patients leads to increased plasma level of IL-33.

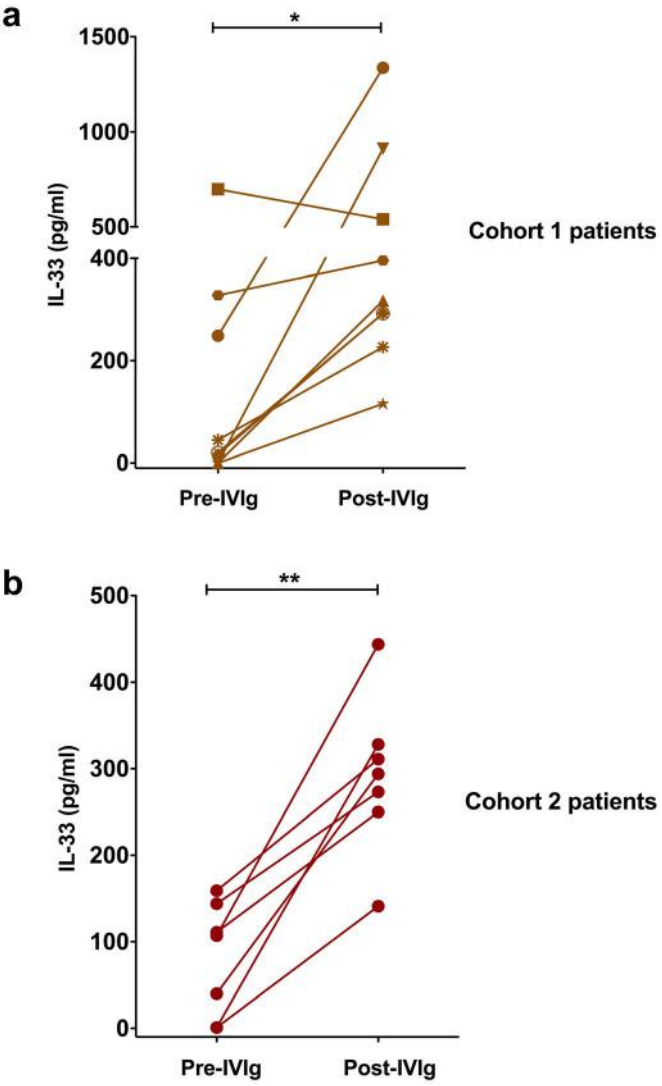

Figure 1 | Consequence of IVIg therapy in autoimmune patients on the plasma level of IL-33. (a) Heparinized blood samples were obtained from nine patients with inflammatory myopathies (Cohort 1 patients) before (Pre-IVIg) and 2-3 days after initiation of IVIg therapy (Post-IVIg). IL-33 (pg/ml) in the plasma was measured by ELISA. Each symbol in the graph represents individual patient. (b) IL-33 in the plasma of four inflammatory myopathies and three anti-neutrophil cytoplasmic antibody-associated vasculitis patients (Cohort 2 patients) before and post-IVIg therapy. The statistical significance as determined by two-tailed Student-t-test is indicated, where *, $P < 0.05$; **, $P < 0.01$.

IVIg-induced IL-33 is not associated with an expansion of basophils. Basophils play a crucial role in the induction of Th2 responses[23,24]. Recent data from K/BxN serum-induced murine arthritis model suggest that IVIg-induced IL-33 promotes basophil expansion[22]. Therefore, we investigated changes in the circulating basophils following IVIg therapy in cohort 1 patients. Basophils were identified based on the expression of FcεRI and CD203c (Fig. 2a)[25]. In contrast to the results from murine model, we found that IVIg therapy leads to basophil expansion only in two patients who also showed increased plasma level of IL-33 (Fig. 2b). In other patients, basophils were either declined or unaltered. The changes in the proportion of basophils in the circulation following IVIg therapy were not statistically significant. Importantly, the fold-changes in IL-33 and basophils were not correlated (Fig. 2c). Also contrary to previous report[22], we could hardly detect IL-4 in the plasma of patients following IVIg therapy. Thus, these results demonstrate that IVIg therapy in patients does not lead to an expansion of basophils. Of note, a recent data from murine models of collagen antibody-induced arthritis and K/BxN serum transfer arthritis also reveal that therapeutic effect of IVIg is independent of sialylation and basophils[26].

DC-SIGN-positive human innate cells do not produce IL-33 upon IVIg exposure. DC-SIGN$^+$ innate cells (or SIGN-R1$^+$ cells in the murine spleen) were proposed to produce IL-33 upon interaction with α2,6-sialylated Fc fragments of IVIg[22]. By generating humanized DC-SIGN-transgenic mice, the authors found that these transgenic mice express DC-SIGN on DCs, macrophages and monocytes in the blood, bone marrow and spleen. Importantly, higher percentage of monocytes in these transgenic mice expressed DC-SIGN[22].

We analyzed the expression of DC-SIGN in human myeloid cells. Contrary to humanized DC-SIGN-transgenic mice, circulating human monocytes did not express DC-SIGN whereas its expression on macrophages was restricted to M2 type macrophages wherein up to 28% cells were positive for DC-SIGN. We could observe high expression of DC-SIGN (≈100%) only in monocyte-derived DCs (Mo-DCs) (Fig. 3a). In the human spleen, up to 24% splenocytes were positive for DC-SIGN (Fig. 3b).

Therefore, we explored if Mo-DCs secrete IL-33 upon IVIg treatment. In contrast to proposition by Ravetch and colleagues, we could

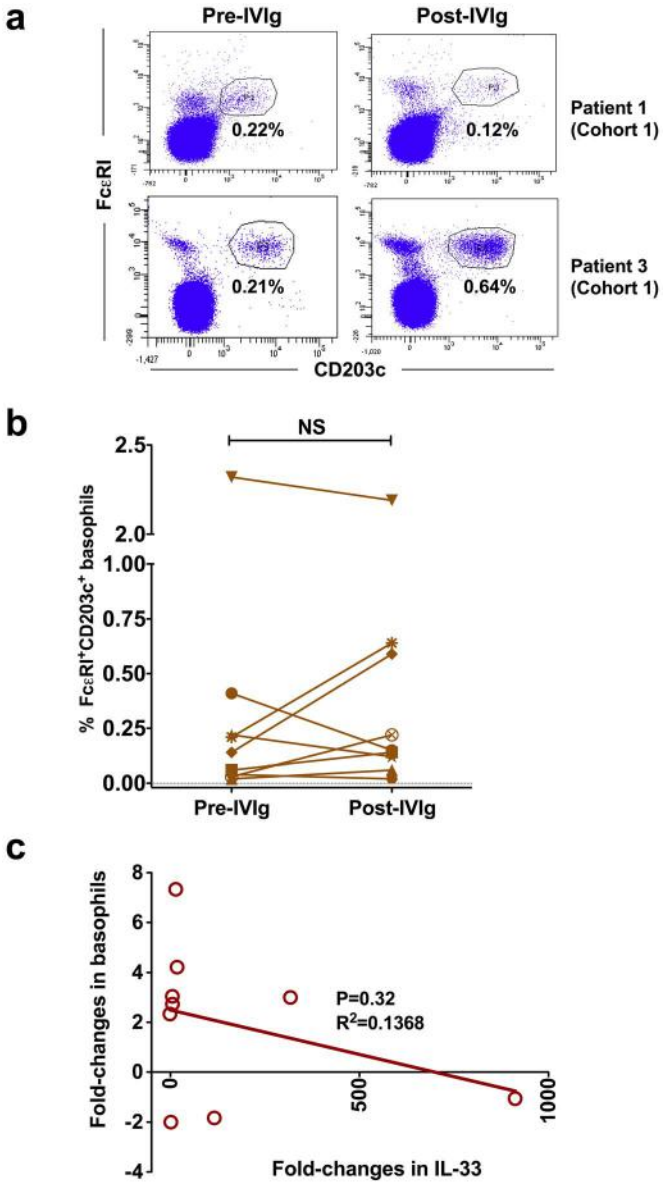

Figure 2 | Changes in the proportion of circulating basophils of autoimmune patients following IVIg therapy. Heparinized blood samples were obtained from cohort 1 patients with inflammatory myopathies before (Pre-IVIg) and 2–3 days after initiation of IVIg therapy (Post-IVIg). (a) Representative dot-plots showing basophils from cohort 1 patients gated positive for FcεRI and CD203c (b) Modulation of circulating basophils following IVIg therapy (n = 9). Basophils were analyzed in the whole blood by flow cytometry. The statistical significance as determined by two-tailed Student-t-test is indicated, where NS, non-significant. (c) The correlation between fold-changes in IL-33 and basophils following IVIg therapy.

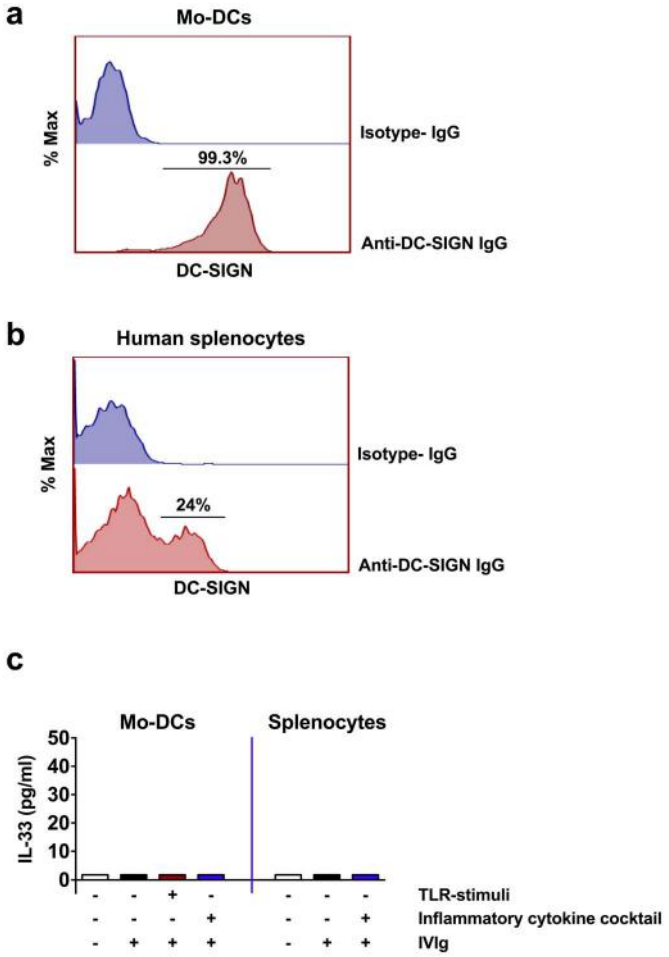

Figure 3 | Effect of IVIg on the IL-33 production from DC-SIGN$^+$ human innate cells. (a and b) Histograms showing the expression of DC-SIGN by healthy donor's monocyte-derived human dendritic cells (Mo-DCs) and splenocytes. (c) IVIg does not induce IL-33 from DC-SIGN$^+$ human innate cells. Mo-DCs or human splenocytes (n = 5 donors) were exposed to IVIg either under non-inflammatory conditions or under inflammatory conditions (TLR-stimuli or inflammatory cytokine cocktail) for 48 hours. IL-33 in the culture supernatants was analyzed by ELISA.

detect secreted IL-33 from IVIg-exposed DC-SIGN$^+$ Mo-DCs neither under non-inflammatory nor under inflammatory conditions (Fig. 3c). Similarly, despite the presence of DC-SIGN$^+$ cells in the spleen, human splenocytes did not produce detectable levels of IL-33 upon IVIg exposure both under inflammatory and non-inflammatory conditions (Fig. 3c).

Discussion

Our results demonstrate that IVIg therapy induces IL-33 in autoimmune patients thus confirming the previous observation made in mice. However, IL-33 was not contributed either by splenic DC-SIGN$^+$ cells or myeloid DCs. Also, the amount of IL-33 induced in the patients was not sufficient to expand basophils. It should be noted that the quantity of IL-33 protein induced in the mice following IVIg treatment was not presented in the previous report. In addition, significant amount of data on IVIg was indirect rather than direct demonstration of IVIg-mediated regulation of cytokine network[22]. Authors showed that IVIg induces about 12-fold increase in IL-33 mRNA level. However, the contribution of this increased IL-33 mRNA towards IL-33 protein is not clear. Considering five liters as total blood volume in adults, our results show that IVIg induces ≈2460 ± 650 ng of IL-33 (based on the data from cohort 1 patients). However, to demonstrate the role of IL-33 in IVIg-mediated anti-inflammatory effects, Anthony *et al.*, injected 400 ng of IL-33 for four consecutive days[22]. As mouse weighing 25 g would have ≈1.5 ml of blood, based on the IL-33 data from patients, we could infer that the amount of exogenous IL-33 injected into the mice represents at least 540-times excess of IL-33 that otherwise induced by IVIg. This might explain why IVIg failed to induce expansion of basophils in the patients. Although in our study, patients' sample size was small, we included diseases such as inflammatory myopathies and vasculitis that were shown to benefit from IVIg therapy. Further investigations in a larger number of patients should confirm these observations.

Table 1 | Summary of data for autoimmune rheumatic patients

Cohort 1 patients

Number	Disease	Age (years)	Sex	IVIg	Additional treatments
1	Polymyositis	59	F	CLAIRYG® 1 g/kg	Methylprednisolone
2	Anti-SRP associated necrotizing myopathy	27	F	CLAIRYG® 1 g/kg	Prednisone, Methotrexate
3	Anti-HMGCR associated necrotizing myopathy	62	F	CLAIRYG® 0.5 g/kg	Prednisone, Methotrexate
4	Anti-HMGCR associated necrotizing myopathy	61	F	CLAIRYG® 1 g/kg	Prednisone, Methotrexate
5	Dermatomyositis	52	F	CLAIRYG® 1 g/kg	Prednisone, Methotrexate
6	Polymyositis associated with mixed connective tissue disease and Sjögren's syndrome	41	F	CLAIRYG® 1 g/kg	Prednisone, Methotrexate
7	Anti-SRP associated necrotizing myopathy	40	M	CLAIRYG® 1 g/kg	Prednisone, Methotrexate
8	Anti-Mi2 associated unclassified myositis	30	M	CLAIRYG® 1 g/kg	Prednisone, Methotrexate
9	Polymyositis and probable associated Sjögren's syndrome	70	F	CLAIRYG® 1 g/kg	Prednisone, Methotrexate

Cohort 2 patients

Number	Disease	Age (years)	Sex	IVIg	Additional treatments
1	Dermatomyositis	22	F	TEGELINE® 1g/kg	Prednisone, Mycophenolate mofetil
2	Polymyositis	42	M	TEGELINE® 1g/kg	Prednisone, Methotrexate
3	Dermatomyositis	35	M	TEGELINE® 1g/kg	Prednisone
4	Polymyositis	46	F	TEGELINE® 1g/kg	Prednisone, ciclosporin
5	Microscopic polyangiitis	61	F	TEGELINE® 1g/kg	Prednisone
6	Wegener's granulomatosis	62	M	TEGELINE® 1g/kg	None
7	Microscopic polyangiitis	61	M	TEGELINE® 1g/kg	Prednisone, Mycophenolate mofetil

SRP, Signal Recognition Particle; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

The role of Fc-sialylation, DC-SIGN and Fcγ receptor IIB (FcγRIIB) in the anti-inflammatory effects of IVIg has been debated recently by several groups[27]. Mice and humans show wide variations in the expression pattern of FcγRs, and the phenotype and anatomical distribution of innate cells. Unlike mice, human innate cells express both activating FcγRIIA and inhibitory FcγRIIB. Therefore, the proposition that IVIg enhances FcγRIIB on effector macrophages of mice without having corresponding data on FcγRIIA might provide a biased picture on the mechanisms of IVIg. Gene array analysis could not confirm IVIg-mediated up-regulation of FcγRIIB in the patients with Kawasaki disease[28]. In line with this report, another recent study failed to demonstrate enhanced expression of FcγRIIB on monocytes following IVIg therapy in children with immune thrombocytopenia[29]. Also, FcγR polymorphisms did not predict response to IVIg in myasthenia gravis[30]. Although DC-SIGN promoter −336 A/G (rs4804803) polymorphism was associated with susceptibility of Kawasaki disease, this variant was found to be not associated with the occurrence of IVIg resistance[31]. Of note, treatment response in Kawasaki disease is apparently associated with sialylation levels of endogenous IgG but not therapeutic IVIg[32]. All these data thus questions the relevance of DC-SIGN-FcγRIIB pathway of anti-inflammatory mechanisms of IVIg in humans.

Several recent studies have challenged the concept of α2,6-sialylated Fc fragments-mediated anti-inflammatory mechanism of IVIg both in experimental models and in humans. IVIg could inhibit human Th17 cell differentiation and expansion independent of antigen presenting cells and hence independent of interaction of DC-SIGN and α2,6-sialylated Fc fragments[13–15]. Also, $F(ab')_2$ fragments of IVIg exerted similar effects thus pointing towards dispensability of α2,6-sialylated Fc fragments in mediating the suppression of Th17 cells. We have demonstrated that DC-SIGN and α2,6-sialylated Fc fragment interaction is dispensable for the anti-inflammatory activity of IVIg on human DCs[33]. $F(ab')_2$ fragments but not Fc fragments of IVIg were shown to mediate Treg expansion by inducing cyclooxygenase-2-mediated prostaglandin E2 secretion in human myeloid DCs and was dependent in part on DC-SIGN[19]. Similarly, sialylation-enriched $F(ab')_2$ fragments could inhibit interferon-α production from toll-like receptor (TLR)7 and TLR9 stimulated human plasmacytoid DCs, although sialic acid itself was not required[34].

In the previous reports, Ravetch and colleagues enriched sialic acid-containing IgG-Fc by using sialic acid-specific lectin *Sambucus nigra* agglutinin-based affinity fractionation[22,35–37]. However, by using same fractionation method, Guhr *et al*., showed that IVIg fractions depleted for the sialylated antibody fraction exert benefits in a murine model of passive-immune thrombocytopenia similar to that of intact IVIg. However, sialic acid-enriched IVIg fraction failed to enhance platelets count in this model[38]. Similar sialic-acid independent anti-inflammatory mechanisms were also reported in murine herpes simplex virus encephalitis model[39]. Further, Käsermann and colleagues showed that lectin fractionation of IVIg results in increased sialylation of Fab fragments but not Fc fragments. By using human whole blood stimulation assay either with lipopolysaccharide or phytohaemagglutinin, they further showed that anti-inflammatory effects of IVIg is associated with $F(ab')_2$ fraction of IVIg[40]. In animal models of immune thrombocytopenia and multiple sclerosis, the beneficial effects of IVIg were independent of Fc or $F(ab')_2$ -sialylation and FcγRIIB[41–44]. Based on these results, it was suggested that genetic background of the mice and dose of IVIg are the critical factors that determine the role of FcγRIIB in IVIg-mediated beneficial effects. In line with these observations, two studies have failed to demonstrate the direct interaction between sialylated IgG Fc fragment and DC-SIGN[45,46]. These data thus point out that α2,6-sialylated Fc fragment-DC-SIGN-FcγRIIB mechanism merely represents one of the several anti-inflammatory mechanisms of IVIg that were reported. Therefore, this anti-inflammatory pathway of IVIg might be operational in certain pathologies and experimental models and might not be considered as a universal mechanism.

It was proposed that in humanized DC-SIGN-transgenic mice, DC-SIGN$^+$ innate cells such as monocytes, macrophages and DCs produce IL-33 upon interaction with α2,6-sialylated Fc fragments of IVIg[22]. Recent reports show that IL-33 is an important player for the promotion of Th2 responses and activated DCs are one of the sources of this cytokine[47,48]. We found that unlike monocytes from huma-

nized DC-SIGN-transgenic mice that were highly positive for DC-SIGN, human monocytes hardly express DC-SIGN. Further, human Mo-DCs despite expressing DC-SIGN, failed to produce IL-33 when exposed to IVIg either under non-inflammatory or inflammatory conditions. In wild type mice, it was suggested that α2,6-sialylated Fc fragments bind to SIGN-R1 expressed on splenic marginal zone macrophages[35]. Marginal zone macrophages are absent in human spleen and data from humans show that spleen is dispensable for the anti-inflammatory effects of IVIg. In line with this concept, by using a passive model of induced immune thrombocytopenia, it was shown that IVIg is fully functional in splenectomized mice although this report supported the sialic acid and SIGN-R1-dependent mechanisms of IVIg[49]. We found that despite the presence of DC-SIGN$^+$ innate cells in the human spleen, IVIg could not induce IL-33 from the splenocytes. All these data indicate that spleen and DC-SIGN$^+$ cells are dispensable for IVIg-mediated IL-33 induction in humans. Thus, the source of IL-33 in humans following IVIg therapy remains elusive. As IVIg is known to cause apoptosis of cells, we suggest that secondary necrosis of late stage apoptotic cells could release IL-33[50–52]. This process might depend on the signals provided by anti-Fas IgG or anti-Siglec IgG in the IVIg preparations rather than the repercussion of interaction of α2,6-sialylated Fc fragments with DC-SIGN[53,54]. In addition, IL-33 is also constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*[55].

Methods

Patients. All experiments were performed in accordance with relevant guidelines and regulations. We obtained heparinized blood samples of nine patients (cohort 1 patients) with inflammatory myopathies (Table 1). Patients were aged 49.1 ± 15.2 years and include two men. Blood samples were obtained before and 2–3 days following initiation of IVIg therapy (CLAIRYG®, Laboratoire Français du Fractionnement et des Biotechnologies, France). Informed consent was obtained from all the patients. The study was approved by CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris. In addition, we also analyzed plasma samples of seven rheumatic patients (cohort 2 patients) before and 2–3 days post-IVIg therapy (TEGELINE®, Laboratoire Français du Fractionnement et des Biotechnologies). The patients were aged 47 ± 5.8 years (four men) and include inflammatory myopathies and anti-neutrophil cytoplasmic antibody-associated vasculitis (Table 1).

Analysis of basophils. Red blood cells (RBCs) from heparinized blood samples of cohort 1 patients were depleted by using HetaSepTM (Stemcell Technologies Sarl, France) and nucleated cell suspension was obtained. Basophils were analyzed in RBC-depleted cell suspension by flow cytometry (LSR II, BD Biosciences, France) using fluorochrome-conjugated monoclonal antibodies to FcεRI (Miltenyi Biotec, France) and CD203c (eBioscience, France). Data were analyzed by FACSDiva™ software (BD Biosciences).

Generation of monocyte-derived DCs. Buffy coats from the healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang (EFS), Paris, France. Institut National de la Santé et de la Recherche Médicale-EFS ethical committee permission (N°12/EFS/079) was obtained for the use of buffy coats of healthy donors. Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coats by density gradient centrifugation using Ficoll-paque PREMIUM (GE healthcare, France). CD14$^+$ monocytes were isolated from PBMCs by using CD14 microbeads (Miltenyi Biotec). Purified monocytes were then cultured for 6 days in RPMI-1640 medium plus 10% fetal calf serum (FCS) containing cytokines GM-CSF (1000 IU/10^6 cells) and IL-4 (500 IU/10^6 cells) (both from Miltenyi Biotec) to obtain DCs[56]. The purity of DCs was >98%. DC-SIGN expression on Mo-DCs was examined by flow cytometry using fluorochrome-conjugated monoclonal antibodies (BD Biosciences) and data were analyzed by FACSDiva™ and FlowJo softwares (Tree Star, USA).

Isolation of human splenocytes. The remnant human spleen sections from individuals submitted for pathological diagnosis were obtained from service d'anatomie pathologique, Hôpital Européen Georges Pompidou, Paris, France. Only healthy spleen tissues were used for the research purpose. Since the study did not require additional sampling, an approval from an ethics committee was not required under French law according to the article L.1121-1 of the public health code. The article states that: The research organized and performed on human beings in the development of biological knowledge and medical research are permitted under the conditions laid down in this book and are hereinafter referred to by the term "biomedical research". The article further states that it does not imply under conditions: For research in which all actions are performed and products used in the usual way, without any additional or unusual diagnostic procedure or surveillance.

The spleen sections were collected in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Single-cell suspension of splenocytes was obtained by mechanical disaggregation of spleen tissue pieces by using gentleMACS dissociator (Miltenyi Biotec) followed by filtration through 70-μm nylon membrane filter (BD Biosciences). Splenocytes were then subjected to Ficoll-Paque PREMIUM density gradient centrifugation to obtain mononuclear cells. DC-SIGN expression on the splenocytes was investigated by flow cytometry using fluorochrome-conjugated monoclonal antibodies and data were analyzed by FACSDiva™ and FlowJo softwares.

Stimulation of cells. Mo-DCs (0.5 × 10^6/ml) were cultured in RPMI 1640-10% FCS containing GM-CSF and IL-4 in a 12-well plate. The cells were then exposed to IVIg (25 mg/ml) for 48 hours to analyze the effect of IVIg on IL-33 production under non-inflammatory conditions. In parallel, Mo-DCs were stimulated with either TLR4 ligand lipopolysaccharide (100 ng/ml/0.5 × 10^6 cells) (Sigma-Aldrich, France) or inflammatory cytokine cocktail (10 ng/ml each of IL-1β, IL-6 and TNF-α, all from ImmunoTools, Germany)[57]. After four hours, IVIg was added and cultures were maintained for 48 hours to analyze the effect of IVIg on IL-33 production under inflammatory conditions.

Similarly, splenocytes (0.5 × 10^6/ml) were cultured in RPMI 1640-10% FCS for 48 hours either alone or with IVIg. In addition, splenocytes were also stimulated with inflammatory cytokine cocktail and IVIg was added to the cultures after four hours. The cultures were maintained for 48 hours.

Quantification of cytokines. IL-33 in the plasma samples of the patients and in cell-free culture supernatants was quantified by ELISA (R&D systems, France). IL-4 in the plasma was also measured by ELISA (R&D systems).

Statistical analysis. Data was analyzed using Prism 5 software (GraphPad software). Two-tailed Student's t-test was used to determine the statistical significance of the data. Values of $P < 0.05$ were considered as statistically significant.

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Author contributions

J.B. designed the research, M.S., C.S., P.H., M.S.M., E.S-V., L.G. & M.L. performed the experiments, M.S., P.H., M.S.M., S.V.K. & J.B. analyzed the data, Y.S., L.M. & O.B. provided blood samples of the patients, P.B. provided the spleen tissues, J.B. wrote the paper and all authors reviewed and approved the manuscript.

Additional information

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Article

Differential Effects of *Viscum album* Preparations on the Maturation and Activation of Human Dendritic Cells and CD4$^+$ T Cell Responses

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Abstract: Extracts of *Viscum album* (VA); a semi-parasitic plant, are frequently used in the complementary therapy of cancer and other immunological disorders. Various reports show that VA modulates immune system and exerts immune-adjuvant activities that might influence tumor regression. Currently, several therapeutic preparations of VA are available and hence an insight into the mechanisms of action of different VA preparations is necessary. In the present study, we performed a comparative study of five different preparations of VA on maturation and activation of human dendritic cells (DCs) and ensuing CD4$^+$ T cell responses. Monocyte-derived human DCs were treated with VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A. Among the five VA preparations tested VA Qu Spez, a fermented extract with a high level of lectins, significantly induced DC maturation markers CD83, CD40, HLA-DR and CD86, and secretion of pro-inflammatory cytokines such as IL-6, IL-8, IL-12 and TNF-α. Furthermore, analysis of T cell cytokines in DC-T cell co-culture revealed that VA Qu Spez significantly stimulated IFN-γ secretion without modulating regulatory T cells and other CD4$^+$ T cytokines IL-4, IL-13 and IL-17A. Our study thus delineates differential effects of VA preparations on DC maturation; function and T cell responses.

Keywords: *Viscum album*; innate cells; dendritic cells; maturation; cytokines; T cell response; IFN-γ; Th17; Th1; Th2; regulatory T cell

1. Introduction

Extracts of *Viscum album* L. (VA) or European mistletoe, a semi-parasitic plant, are traditionally used for the complementary therapy of cancer and other disorders [1–4]. Several lines of evidence indicate that VA improves patient survival, reduces the damage caused by conventional cancer therapies and increases patients' quality of life [1,5,6]. Depending on the concentration used for treatment, mistletoe extracts induce tumor cell death and exert direct necrotic effects or apoptosis [2]. VA preparation is a heterogeneous mixture of several bio-active molecules, but the major components are lectin and viscotoxin. Mistletoe lectin (ML) consists of two subunits, the A chain (29 KDa) and B

chain (34 KDa). The A chain is responsible for ribosome inactivation, whereas the B chain helps in binding to terminal galactoside residues on cell membrane [7,8].

Dendritic cells (DCs) are antigen presenting (APCs) and involved in mounting and modulating the immune response. Being sentinels of the immune system, DCs bridge innate and adaptive immunity. Thus, DCs are potential targets for the therapeutic intervention in immune-mediated conditions. Immature DCs expressing low MHC II on their surface are specialized in uptake of antigens. Upon receiving activation signals, DCs undergo maturation and induce distinct $CD4^+$ T cell responses. The mature DCs express high level of MHC II and co-stimulatory molecules and secrete a large array of cytokines that mediate inflammation and $CD4^+$ cell polarization [9–14]. However, in the absence of danger signals, presentation of self-antigens by immature DCs promotes immune tolerance by silencing the effector and autoreactive T cells and enhancing $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) or T regulatory type 1 cells [9,15–18].

As DCs have a central role in anti-tumor immune responses, efficient functioning of these cells is crucial for the success of cancer immunotherapy [19]. DCs are immature and functionally defective in cancer patients and tumor-bearing animals, possibly due to insufficient danger signals in the tumor microenvironment [20]. Further, several reports indicate that tumor cells hamper the maturation process of DCs and their capacity to prime protective T cell responses [21–24].

Our previous report demonstrates that VA Qu Spez, one of the VA preparations, induces activation of human DCs, and DC-mediated $CD4^+$ T cell proliferation and tumor-specific $CD8^+$ T cell responses as measured by IFN-γ and TNF-α secretion [25]. However, several therapeutic preparations of VA are currently available. Each VA preparation is heterogeneous in its chemical composition and is influenced by the host tree, harvest season and extraction method [26–28]. Therefore, the therapeutic outcome of a particular VA preparation might not be similar to that of other preparations [29,30]. An insight into the mechanisms of action of different VA preparations is therefore necessary to provide guidelines for the correct therapeutic use of VA preparations.

In the present study, we performed a comparative study of five different preparations of VA (VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A) on the maturation and activation of human DCs and ensuing $CD4^+$ T cell responses. Our data show that among five preparations tested, VA Qu Spez is the most potent inducer of DC maturation and secretion of DC cytokines. Furthermore, VA Qu Spez significantly stimulated IFN-γ secretion without modulating Tregs and other $CD4^+$ T cytokines IL-4, IL-13 and IL-17. Our study thus delineates differential effects of VA preparations on DC maturation, function and T cell responses.

2. Results

2.1. Effect of Different VA Preparations on the Maturation of DCs

Immature DCs of 5 day old were either untreated or treated with five VA preparations at four different concentrations: 5, 10, 15 and 20 µg/mL/0.5 $\times$ 10^6 cells for 48 h. DCs were analysed for the expression of various maturation-associated surface molecules (Figure 1A–F). We found that among five VA preparations, only VA Qu Spez was able to significantly enhance the intensity of expression of antigen presenting molecule HLA-DR, co-stimulatory molecules CD86 and CD40 and % of expression of terminal maturation marker CD83. The induction of DC maturation by VA Qu Spez was observed only at higher concentrations i.e., 15 and 20 µg. Further, the effect of VA Qu Spez on maturation of DCs was dose-dependent. The expressions of CD40 and HLA-DR were 100% on control DCs and were not altered by VA Qu Spez. VA Qu Spez also did not alter % expression of CD1a and intensity of expression of CD83.

We observed that HLA-DR expression on VA Qu Spez (20 µg) and LPS (positive control, 10 ng/0.5 million cells)-stimulated DCs was similar. However, induction of CD40 and CD86 by VA Qu Spez was 2-fold lesser and CD83 was 4-fold lesser than LPS. In line with our previous report on stimulation of tumor-antigen-specific cytotoxic T cell responses by VA Qu Spez-stimulated DCs [25], we found

that these DCs expressed higher levels of HLA class I molecules (13.6% ± 1.1% on control DCs vs. 20.6% ± 3.2% on VA Qu Spez-stimulated DCs, $n = 3$). However, VA Qu Frf, VA M Spez, VA P and VA A did not significantly modify the expressions of any of maturation-associated molecules on DCs. These results suggest that among all preparations tested; only VA Qu Spez is able to induce maturation of DCs.

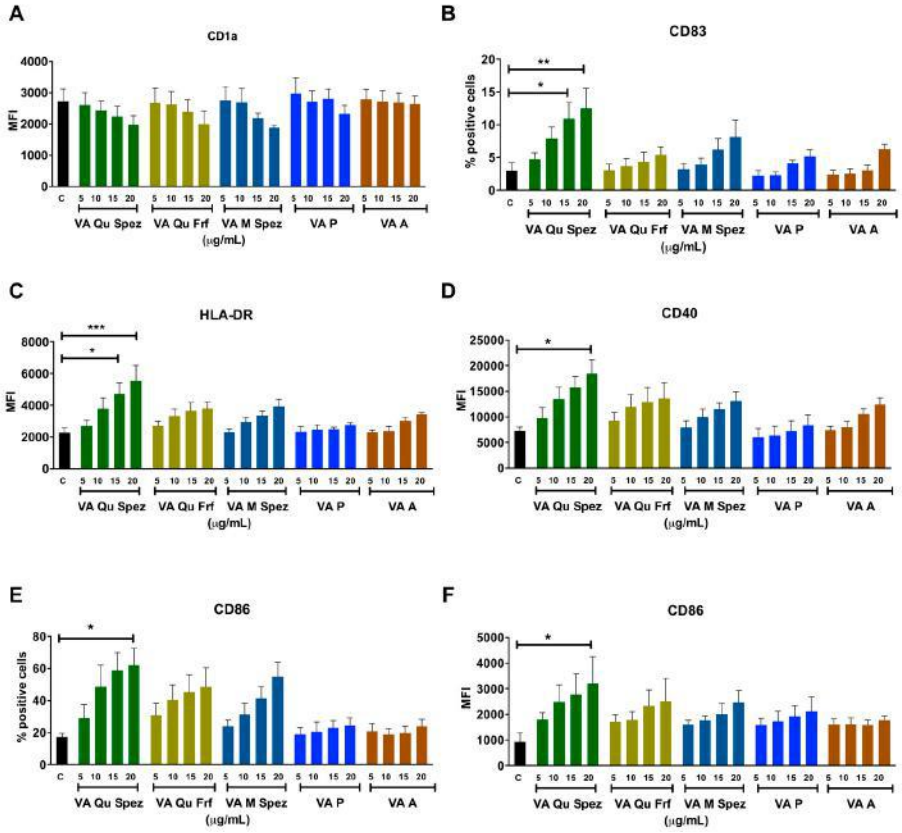

Figure 1. Differential effects of VA preparations on the phenotype of human DCs. Immature DCs were treated with medium alone (control, labelled as 'C') or with five preparations of VA (VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A) at indicated concentrations for 48 h. Expressions (mean ± SEM, ⩾4 independent donors) of (**A**) CD1a; (**B**) CD83; (**C**) HLA-DR; (**D**) CD40; (**E**,**F**) CD86 on DCs were analysed by flow cytometry. The data are presented either as % positive cells or MFI of indicated markers. X-axis denotes concentrations of VA preparations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.2. VA Qu Spez but Not Other VA Preparations Stimulate the Secretion of DC Cytokines

It is well reported that DC-derived cytokines play a critical role in regulating the immune responses and in polarizing distinct $CD4^+$ T cell responses. We analysed the differential effects of various VA preparations on the secretion of DC cytokines such as IL-6, IL-8, IL-12, IL-10 and TNF-α. As VA Qu Spez significantly induced maturation of DCs, it was likely that this effect is associated with modulation of DC cytokines. In fact, compared to control DCs, VA Qu Spez-treated DCs showed significantly increased secretion of IL-6, IL-8, IL-12 and TNFα (Figure 2A–C,E). Control DCs secreted 4.7 ± 5.1 pg/mL of IL-6 and was enhanced to 156.9 ± 105.1 pg/mL by VA Qu Spez. In case of IL-8, control DCs secreted 102.2 ± 78.5 pg/mL, whereas VA Qu Spez at the highest concentration induced 612.1 ± 20.4 pg/mL. The Th1-polarizing cytokine IL-12 was secreted at 3.3 ± 4.9 pg/mL by control DCs and was increased to 10.4 ± 6 pg/mL by VA Qu Spez-treated DCs. TNF-α secretion by untreated DCs was 3.2 ± 2.1 pg/mL, and with VA Qu Spez treatment, this cytokine was increased to 135.7 ± 37.9 pg/mL. We could observe a moderate but insignificant induction of the aforementioned DC cytokines by VA Qu Frf and VA M Spez. However, VA P and VA A did not modulate any of the DC cytokines (Figure 2A–C,E). These results show that VA Qu Spez is the most potent

preparation that induces both maturation and cytokines by DCs. Of note, production of IL-10, an immunosuppressive cytokine was unaltered upon VA Qu Spez treatment (Figure 2D). Together, our data suggest that VA Qu Spez significantly induces several pro-inflammatory cytokines without modulating immune-suppressive cytokine IL-10.

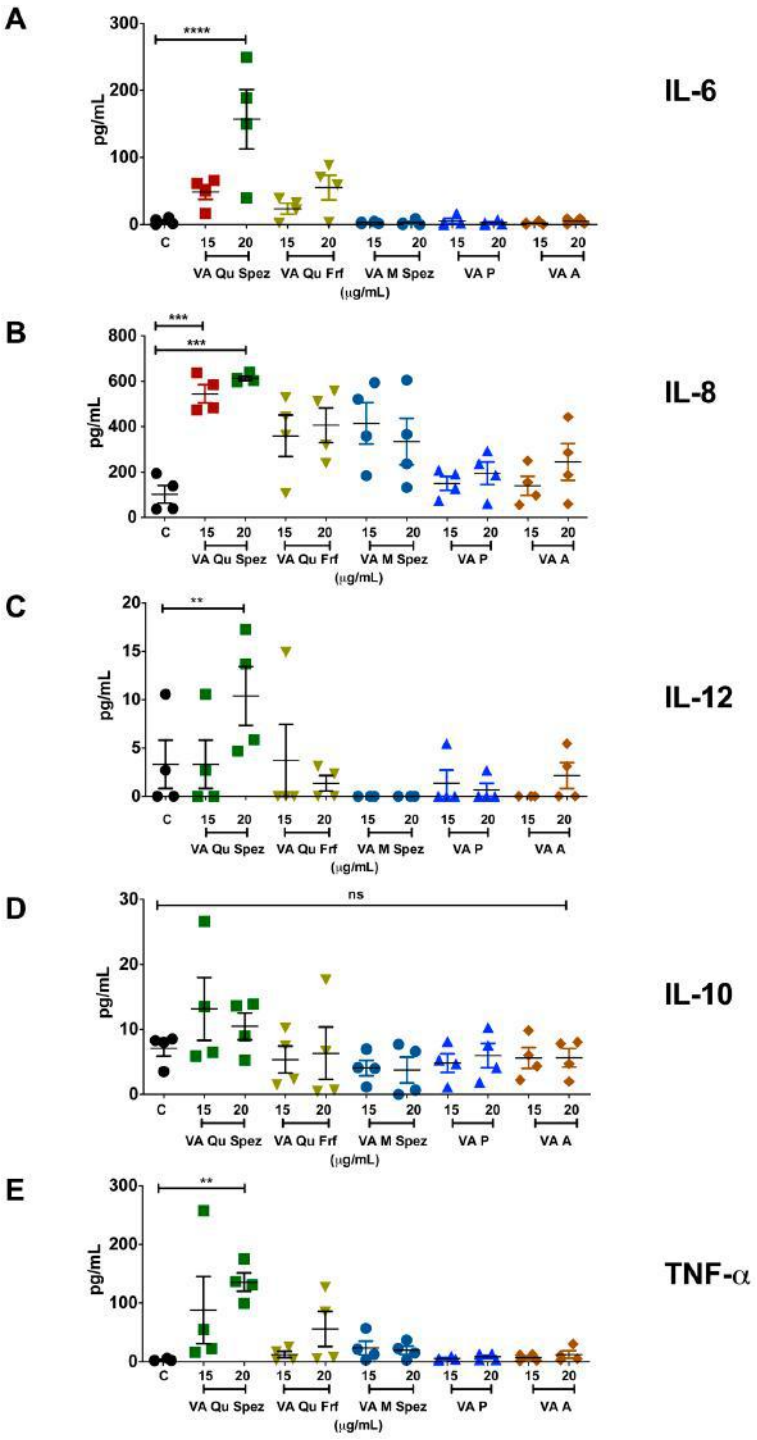

Figure 2. VA Qu Spez but not other VA preparations stimulate the secretion of DC cytokines. Immature DCs were untreated (control, labelled as 'C') or treated with five preparations of VA at various concentrations for 48 h. The amount (pg/mL, mean ± SEM, four independent donors) of (**A**) IL-6; (**B**) IL-8; (**C**) IL-12; (**D**) IL-10; and (**E**) TNF-α in cell-free supernatants was measured. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

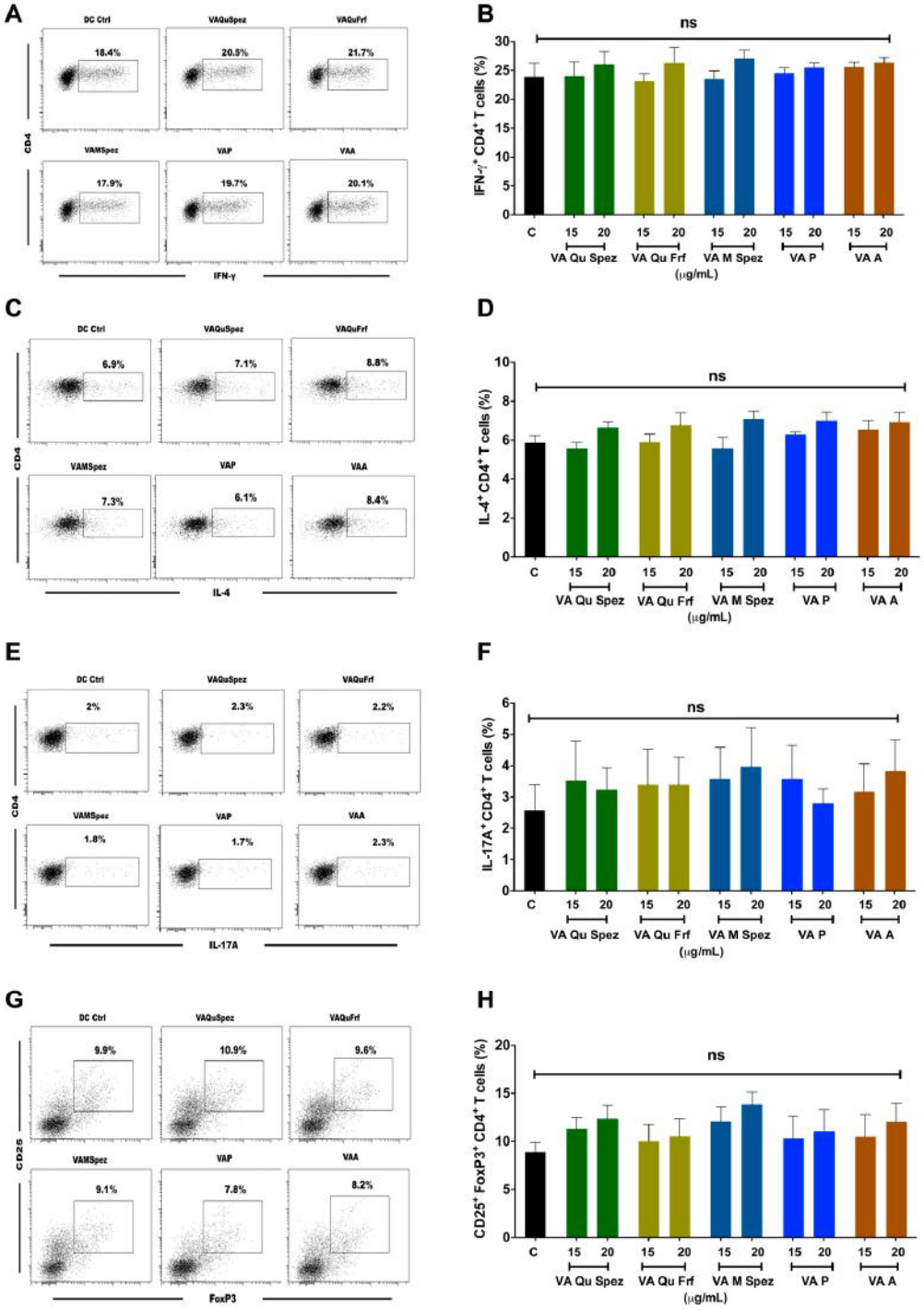

Figure 3. Effect of various VA preparations on the $CD4^+$ T cell responses. DCs were treated with medium alone (DC Ctrl, labelled as 'C') or with five preparations of VA for 48 h. These DCs were co-cultured with $CD4^+$ T cells at 1:10 ratio. After five days of co-culture, the cells were analysed for the various $CD4^+$ T cell subsets by intra-cellular cytokines (IFN-γ, IL-4, IL-17A) or transcription factor (FoxP3) for Th1, Th2, Th17 and Tregs respectively. (**A**,**C**,**E**,**G**) representative dot plots showing the proportion of IFN-γ^+, IL-4^+, IL-$17A^+$ $CD4^+$ T cell and $CD4^+CD25^+Foxp3^+$ T cells respectively; (**B**,**D**,**F**,**H**) Percentage (mean ± SEM, six independent donors) of IFNγ^+ Th1, IL-4^+ Th2, IL-$17A^+$ Th17 and $CD4^+CD25^+Foxp3^+$ Treg cells respectively. ns, non-significant.

2.3. Differential Effects of VA Preparations on the CD4$^+$ T Cell Response

One of the key functions of APC is to promote CD4$^+$ T cell responses. DCs primed with various preparations of VA were co-cultured with CD4$^+$ T cells and Th1, Th2, Th17 and Treg responses were determined by flow cytometric analysis of intracellular IFN-γ (Th1), IL-4 (Th2), IL-17A (Th17), FoxP3 (Treg). Although VA Qu Spez induced maturation of DCs, this effect was not associated with the modulation of frequency of any of the T cell subsets (Figure 3A–H). However, analysis of amount of secretion of T cell cytokines in DC-CD4$^+$ T cell co-culture revealed that VA Qu Spez significantly stimulated IFN-γ secretion (Figure 4A), without having any effect on the secretion of IL-4 (Figure 4B), IL-13 (Figure 4C) and IL-17A (Figure 4D). These results suggest that VA Qu Spez selectively favours Th1 responses without modulating Th2, Th17 and Treg responses. Other four preparations of VA did not alter either frequency of T cell subsets or secretion of various T cell cytokines. These results were in line with the fact that VA Qu Frf, VA M Spez, VA P and VA A did not induce maturation and activation of DCs.

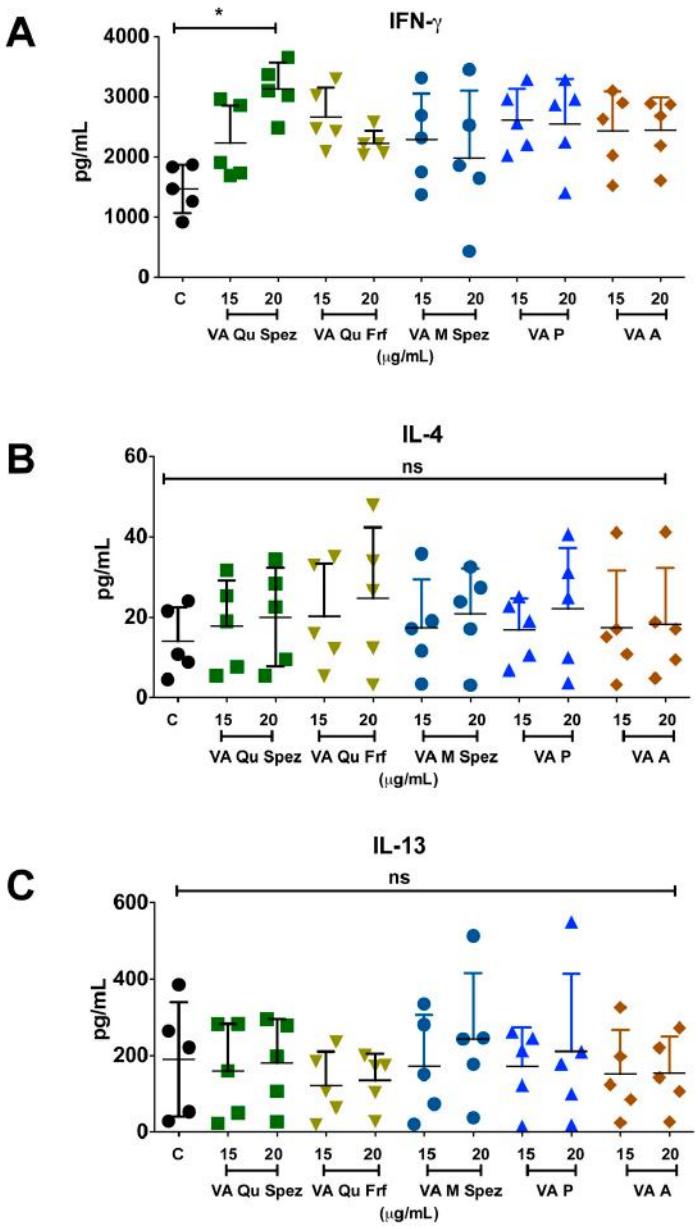

Figure 4. *Cont.*

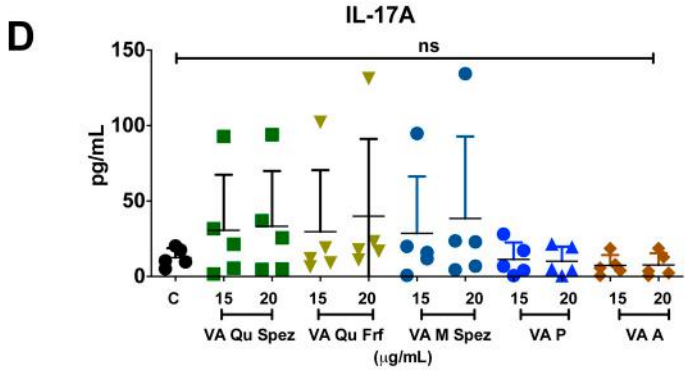

Figure 4. VA Qu Spez-educated DCs significantly induce the secretion of Th1 cytokine IFN-γ in DC-CD4$^+$ T co-cultures. Immature DCs were treated with medium alone (control, labelled as 'C') or with five preparations of VA for 48 h. These DCs were co-cultured with CD4$^+$ T cells for five days. Amount (pg/mL, mean ± SEM, five independent donors) of secretion of (**A**) IFN-γ; (**B**) IL-4; (**C**) IL-13; and (**D**) IL-17A in the cell-free supernatants from the above co-cultures was presented. * $p < 0.05$.

3. Discussion

Currently available mistletoe extracts are highly heterogeneous due to differences in the host trees, nutritional source, season of harvest, and extraction methods [4,26–28]. Therefore, VA preparations could exert divergent biological activities. However, comparative study of immunomodulatory properties of different VA extracts on immunocompetent cells such as DCs has not been performed to date. The present data therefore provide guidelines for the therapeutic use of VA preparations.

IFN-γ plays an important role in mediating the protective immune response against cancer, viral and intracellular bacterial infections [31]. IFN-γ enhances MHC class I expression on tumor cells and MHC class II expression on APCs like DCs, which in turn link innate and adaptive immunity [32]. IFN-γ responsiveness of tumor cell is important for the successful immune recognition. Indeed, it has been demonstrated that mice that are non-responsive to IFN-γ develop more tumors as compared to wild-type mice. Studies have shown that cross-talk between lymphocytes and IFN-γ/STAT1 signalling pathway plays an important role in maintaining the immune competiveness of the host [33]. Idiotype-specific CD4$^+$ Th1 cells can achieve tumor apoptosis directly by Fas/Fas L interaction and indirectly by IFN-γ production [34]. Thus, IFN-γ pathway is considered as an extrinsic tumor-suppressor mechanism [35]. We found that VA Qu Spez significantly enhances IFN-γ production without modulating Treg subsets and production of other T cell cytokines IL-4, IL-13 and IL-17A. This selective enhancement of Th1 cytokine strongly supports the use of VA as an immune modulator.

The success of DC-based cancer immunotherapies is dependent on the maturation status of DCs, their migration capacity and ability to mount protective T cell responses [36]. DC immunotherapy for cancer in humans though shown promises, it has not met with great success as compared to therapeutic molecules that target immune checkpoints. The reasons are multiple including poor survival of transferred DCs, limited number of DCs reaching the secondary lymphoid organs, heterogeneity in the DC subtypes and immune suppressive environment created by the tumor. Previous reports have shown that PGE_2 produced by DCs mediate Treg expansion [37–39], which might help in tumor evasion. Vaccination of cancer patients with 'PGE_2-educated DCs' also induced Treg expansion in the patients [40]. We observed that VA Qu Spez did not modulate Treg responses suggesting that VA Qu Spez selectively induces IFN-γ responses. Although not examined in DCs, we have recently shown that VA Qu Spez inhibits COX2-mediated PGE_2 in epithelial cell line [41,42]. Therefore, it is likely that VA Qu Spez-mediated suppression of COX-2 in DCs might be responsible for nonmodulation of Tregs in the present study. As these data are from the in vitro experiments, further work is necessary to validate these results from the patients treated with VA. Of note, through enhancement of Fas/FasL

expression and caspase activation, IFN-γ has been shown to enhance apoptotic response to ML II in human myeloid U937 cells [43].

MLs are the active components of mistletoe extracts and have several functions. The cytotoxicity of mistletoe is attributed majorly to its lectin contents [44,45] and lectin internalization is required for ML-I-mediated apoptosis [46]. MLs are responsible for stimulating cells of the innate and adaptive immune system such as DCs, macrophages, natural killer cells, and B and T lymphocytes. This function of MLs might represents one of the mechanisms responsible for the anti-tumoral and immunomodulatory effects of mistletoe extracts. It is known that ML-I B chain causes Ca^{2+} influx in Jurkat cells and is mediated by its interaction with surface glycoprotein receptors [47]. Chemical labelling of the lectin revealed that it binds to surface of peripheral and intra-tumoral monocytes [48].

A recent study shows that 3D structure of ML-A chain shares structural homology with shiga toxin from *Shigella dysenteriae* and provides an explanation for the strong immune stimulatory capacity of ML [49]. It is also demonstrated that Korean mistletoe lectin (KML) induces activation of innate cells by TLR4-mediated signalling [50]. The nature of the receptor(s) on DCs that recognizes ML and mediates activation is not known. Since Korean ML and European ML share 84% sequence identity [51], it is presumable that European ML might signal DCs via TLR [49]. However, we found that not all VA preparations are stimulatory on DCs. VA Qu Frf, an unfermented preparation containing the highest concentration of lectin and viscotoxin was unable to activate DCs. Other VA preparations, which are fermented and contain low lectin, were also unable to stimulate DCs, whereas VA Qu Spez, a fermented preparation that contains the second highest concentration of lectin (785 ± 10% ng/mL) efficiently activated DCs and promoted Th1 response. These results suggest that mere lectin content in a VA preparation does not necessarily determine its immunostimulatory capacity. The methodology of preparation, i.e., fermented vs unfermented, might be crucial for conferring the stimulatory properties to VA. Alternatively, the fermentation process might modify the structure of the lectins of the VA preparation.

To conclude, our study delineates the differential effects of various VA preparations on DC maturation, function and T cell responses. These results reveal that VA Qu Spez is the most potent preparation in activating DCs and promoting Th1 response. The current evidence to support mistletoe therapy in oncology is weak [52]. Thus, this study along with other reports on mistletoes [53–60] provides a rational for examining the use VA as an immune modulator. Such mechanistic studies are also important to undertake randomised clinical trials to improve level of evidence for the use of VA in complementary therapy of cancer.

4. Materials and Methods

4.1. VA Preparations

Five clinical grade preparations of VA (VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A) obtained from Hiscia Institute, Verein für Krebsforschung (Arlesheim, Switzerland) were used. These preparations were free from endotoxins and were formulated in 0.9% sodium chloride isotonic solution as 5 mg/mL vials. The chemical compositions of the VA preparations are provided in Table 1.

Table 1. Composition of VA preparations.

Preparation Concentration	Host Trees	Lectin Content (ng/mL)	Viscotoxin Content (μg/mL)	Method of Preparation
VA Qu Spez 10 mg	Quercus (Oak)	785 ± 10%	5 ± 5%	Fermented
VA Qu Frf 10 mg	Quercus (Oak)	2391 ± 10%	19 ± 5%	Unfermented
VA M Spez 10 mg	Malus (Apple)	548 ± 10%	4 ± 5%	Fermented
VA P 10 mg	Pinus (Pine)	28 ± 10%	6 ± 5%	Fermented
VA A 10 mg	Abies (Fir)	23 ± 10%	19 ± 5%	Fermented

4.2. Human DCs

Human monocyte-derived DCs were used as a source of DCs. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors. The buffy coats were purchased from Centre Necker-Cabanel (EFS, Paris, France). Ethics committee approval for the use of such material (Institut National de la Santé et de la Recherche-EFS Ethical Committee Convention N°12/EFS/079) was obtained and experiments were performed in accordance with the approved guidelines of INSERM. Circulating monocytes were isolated using CD14 microbeads (Miltenyi Biotec, Paris, France) and were cultured for 5 days in RPMI 1640 containing 10% fetal calf serum, rhIL-4 (500 IU/10 ^{6} cells) and rhGM-CSF (1000 IU/10^6 cells) to obtain immature DCs [61].

4.3. Viscum Album Treatment of DCs

Immature DCs were washed and cultured in rhIL-4 and rhGM-CSF and treated with VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A at four different concentrations: 5, 10, 15 and 20 µg/mL/0.5 million cells for 48 h. Cell culture supernatants were collected for analysing the cytokines and DCs were analysed for the phenotype by flow cytometry.

4.4. DC: CD4$^+$ T Cell Co-Cultures

CD4$^+$ T cells were isolated from the PBMC using CD4 microbeads (Miltenyi Biotec). VA-treated DCs were washed extensively and seeded with 1 × 10^5 responder allogeneic CD4$^+$ T cells at DC: T cell ratio of 1:10. On 5th day, CD4$^+$ T cell responses were analysed by intra-cellular staining for specific T cell cytokines (IFN-γ, IL-17A and IL-4) and transcription factor (FoxP3). The cell-free culture supernatants were analysed for the cytokines secreted.

4.5. Flow Cytometry

For surface staining, following Fc receptor blockade, antibodies against surface molecules were added at pre-determined concentration and incubated at 4 °C for 30 min. FITC-conjugated monoclonal antibodies (MAbs) to CD1a, CD86, HLA-DR, and CD25; PE-conjugated MAbs to CD83 (all from BD Biosciences, Le Pont de Claix, France), CD40 (Beckman Coulter, Villepinte, France) and Alexa Fluor® 700-conjugated MAbs to CD4 (eBioscience, Paris, France) were used for the analysis of surface phenotype.

For intra-cellular staining, cells were stimulated with phorbolmyristate acetate (50 ng/mL; Sigma-Aldrich, St. Quentin Fallavier, France) and ionomycin (500 ng/mL; Sigma-Aldrich) at 37 °C for 5–6 h in the presence of golgi-stop (BD Biosciences) during the last 2 h. Cells were fixed and permeabilised using Foxp3 Fixation/Permeabilization kit (eBioscience) and incubated at 4 °C with FITC-conjugated MAbs to IFN-γ (eBioscience), PE-conjugated MAbs to IL-17A and IL-4 (eBioscience), and APC-conjugated MAbs to FoxP3 (eBioscience). Live-dead cells were differentiated by PO-Fixable Viable dye (eBioscience).

Cells were acquired on LSR II and processed with FACS DIVA software (BD Biosciences) and analysed by Flowjo. The data were presented as % positive cells for indicated markers or mean fluorescence intensities (MFI) of their expression.

4.6. Cytokine Assay

IL-6, IL-8, IL-10, IL-12, TNF-α, IL-4, IL-13, IFN-γ and IL-17A in the cell-free culture supernatants were quantified by Ready-SET-Go enzyme-linked immunosorbent assay (ELISA) kits (eBioscience).

4.7. Statistical Analysis

The significant differences between samples were determined by One-way ANOVA Tukey's multiple comparison test using Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Values of $p < 0.05$ were considered statistically correlated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

5. Conclusions

Our study demonstrates the differential effects of various VA preparations on human DC activation and ensuing $CD4^+$ T cell responses. Our data reveal that VA Qu Spez is the most potent VA preparation in activating DCs and promoting Th1 response.

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Author Contributions: Conceived and designed the experiments: Chaitrali Saha, Jagadeesh Bayry and Srini V. Kaveri; Performed the experiments: Chaitrali Saha; Mrinmoy Das and Emmanuel Stephen-Victor; Analyzed the data: Chaitrali Saha, Mrinmoy Das, Emmanuel Stephen-Victor, Alain Friboulet, Jagadeesh Bayry and Srini V. Kaveri; and Wrote the paper: Chaitrali Saha, Jagadeesh Bayry and Srini V. Kaveri.

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