Immune Recognition of Latency-insitigating Pathogens by Human Dendritic Cells

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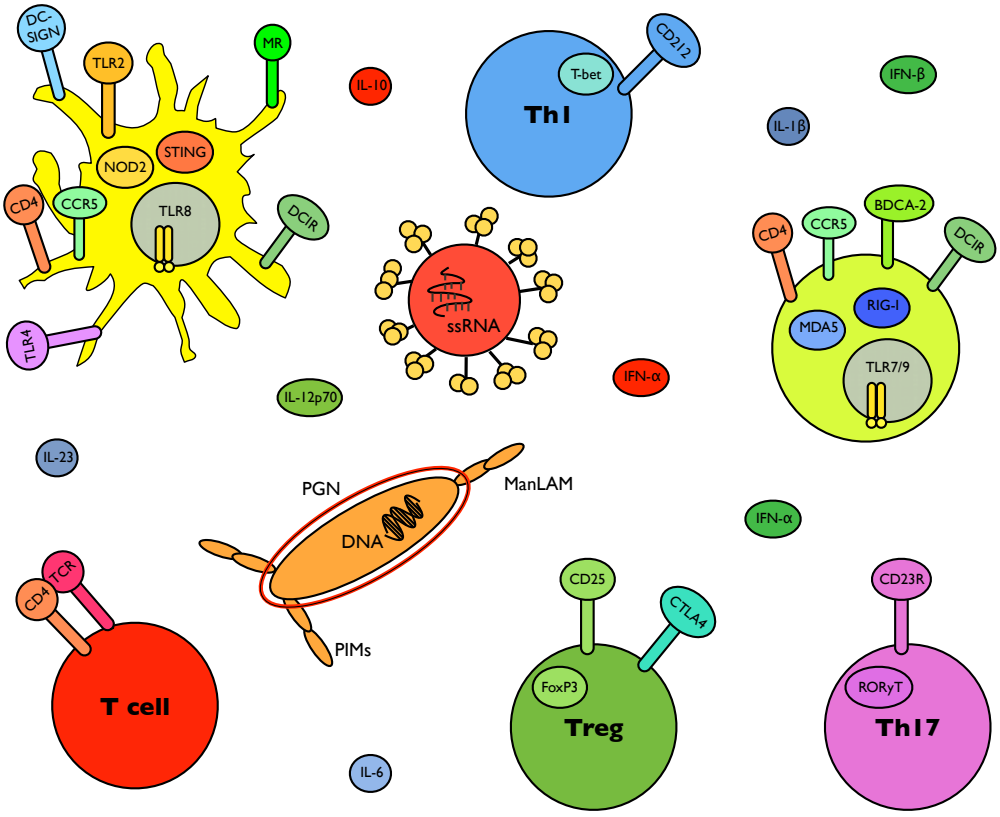

Ph.D. Thesis

Technical University of Denmark

June 2012

Preface

You are currently holding my Ph.D. thesis in your hands. The purpose of this work is to generate a better understanding of how the two notorious pathogens HIV-1 and *M. tuberculosis* are recognized by the immune system. By expanding this knowledge we may be able to generate better treatments for AIDS and Tuberculosis.

This project has been conducted in the Graduate School of Immunology's division at the Technical University of Denmark (DTU), Lyngby, Denmark. Most of the work was done at the Center for Biological Sequence Analysis in the Department of Systems Biology at DTU. I also spend 2 months in the Virus Research and Development group in the Department of Virology at Statens Serum Institut (SSI), Copenhagen, Denmark. The project was funded by the Lundbeck Foundation and the Aase and Ejnar Danielsen's Foundation.

I would like to thank Susanne Brix Pedersen for her helpful guidance in her role as my supervisor at DTU. I would also like to thank Lasse Vinner for being my supervisor at SSI, and Lisbeth Buus Rosholm for helping out with experiments when things got hectic. Finally I would like to thank Erwin M. Schoof and Rune Linding for a good collaboration on the phosphoproteome experiments.

June 2012, Copenhagen, Denmark

Jonas Nørskov Søndergaard

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Abstract

Latent infections with the human pathogenic microorganisms *Mycobacterium tuberculosis* (Mtb) and the human immunodeficiency virus (HIV) are creating some of the most devastating pandemics to date, with great impact on the infected people's lives, their expected lifetime, as well as general costs for society. Consequently there is a pressing need to search for new treatment strategies. Nowadays it is known that HIV-1 and Mtb have acquired the ability to escape the removal from the body by exploiting the immune system for their own benefits. Dendritic cells (DCs) determine the way the immune response unfolds by signaling other immune cells how to respond. An early deregulation of the DCs may therefore propagate into detrimental effects in later stages of the immune response, and may permit HIV-1 and Mtb to become latent. Hence, understanding the way HIV-1 and Mtb interacts with DCs could lead to novel treatment strategies. In the present work this has been examined in purified human plasmacytoid DCs (pDCs) and monocyte-derived DCs (moDCs). First it is demonstrated how Mtb exploits plasticity in moDCs to avoid production of the cytokine IL-12p70 necessary for protection against Mtb. Then it is shown that Mtb induces signaling in moDCs that misdirects the immune response into an extracellular Th17 response, even though the bacteria hide inside immune cells. Finally it is demonstrated how HIV-1 strains, capable of provoking sustained infection, induce a high-mannose-independent complete necrotic eradication of the pDCs that is needed to inhibit initial infection. The results presented in this thesis provide novel insights into immune evasion strategies employed by HIV-1 and Mtb. These findings could eventually be utilized for better treatment strategies against AIDS and tuberculosis disease when specific strategies for immune cell perturbations are established.

Dansk Resumé

Latente infektioner med de humane patogene mikroorganismer *Mycobacterium tuberculosis* (Mtb) og human immundefekt virus (HIV) har skabt nogle af de største pandemier til dato, og har stor indflydelse på de inficerede menneskers liv, deres forventede levetid, samt generelle omkostninger for samfundet. Derfor er der et presserende behov for at søge efter nye behandlingsstrategier. I dag er det kendt, at HIV-1 og Mtb har erhvervet evnen til at undslippe fjernelse fra kroppen ved at udnytte immunresponset til deres egne fordele. Dendritiske celler (DCer) er afgørende for hvordan immunresponset propagerer ved at signalere til andre immunceller, hvordan de skal respondere. En tidlig deregulering af DCerne kan derfor føre til skadelige virkninger i senere faser af immunresponset, og dermed betyde at HIV-1 og Mtb bliver latente. Derfor kan en bedre forståelse af måden hvorpå HIV-1 og Mtb interagerer med DCer føre til hidtil ukendte behandlingsstrategier. I det foreliggende arbejde er dette blevet undersøgt i oprensede humane plasmacytoide DCer (pDCer) og monocyt-afledte DCer (moDCer). Først beskrives hvordan Mtb kan udnytte plasticiteten af moDCer til at forhindre produktion af cytokinet IL-12p70, der ellers er nødvendig for at beskytte mod Mtb. Derefter demonstreres det, at Mtb inducerer signalering i moDCer som medierer et ekstracellulært Th17 respons, selv om bakterien gemmer sig indeni immunceller. Endelig er det vist hvordan HIV-1-stammer, der er i stand til at fremtvinge en vedvarende infektion, inducerer en mannose-uafhængig komplet nekrotisk udryddelse af pDCer, som ellers er nødvendige for at inhibere tidlig infektion. Resultaterne der præsenteres i denne afhandling giver ny indsigt i immunevasionsstrategier som HIV-1 og Mtb benytter. Disse resultater kan på lang sigt anvendes til bedre behandlingsstrategier mod AIDS og tuberkulose, når specifikke strategier for immuncelleperturbering er etableret.

List of original papers included in this thesis

- Søndergaard JN and Brix S. *M. tuberculosis* regulates dendritic cell plasticity towards high CD14 and low IL-12p70 that reprograms upon exogenous IFN-γ. **Submitted to J. Leukoc. Biol.** (2012).
- Søndergaard JN and Brix S. Isolation of IL-12p70 competent human monocyte-derived dendritic cells. **In review J. Immunol. Methods** (2012).
- Søndergaard JN, Schoof EM, Linding R, and Brix S. Kinome-wide analysis of human dendritic cells to deduce pathways involved in *M. tuberculosis*-mediated Th17-bias. **In preparation** (2012).
- Søndergaard JN, Vinner L, and Brix S. High-mannose glycosylation of HIV-1 gp120 and immune regulation in human plasmacytoid dendritic cells. **Submitted to J. Infect. Dis.** (2012).

Abbreviations

Abbreviation	Full name
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AIM2	absent in melanoma 2
ASC	apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain)
AT	aldrithiol
BALF	bronchoalveolar lavage fluid
BCG	The Bacille Calmette-Guérin
BDCA	blood dendritic cell antigen
CCL	chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD40L	CD40ligand
CLR	C-type lectin receptor
CTL	cytotoxic T lymphocyte
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (CD209)
DCIR	dendritic cell immunoreceptor
ds	double-stranded
Endo	Endoglycosidase
ERK	extracellular-signal-regulated kinase
FBS	fetal bovine serum
FoxP3	forkhead box P3
gDNA	genomic DNA
GM-CSF	granulocyte-macrophage colony-stimulating factor
GNL	*Glanthus nivalis*
gp120	glycoprotein 120
HAART	highly active antiretroviral therapy
Hes	hairy and enchancer of split
Hey	hairy/enhancer-of-split related with YRPW motif protein
hi	high
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
iE-DAP	γ-D-glutamyl-meso-diaminopimelic acid
IFN	interferon

IL	interleukin
IPAF	ICE-protease-activating factor
IRAK-M	interleukin-1 receptor-associated kinase 3
IRF	IFN-regulatory factor
IκB	inhibitor of NF-κB
JNK	c-Jun N-terminal kinase
lo	low
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTR	long terminal repeat
ManLAM	mannosylated lipoarabinomannan
MDA5	melanoma-differentiation-associated gene 5
mDC	myeloid dendritic cell
MDP	muramyldipeptid
MDR	multidrug resistant
MEK	mitogen activated protein kinase/ERK kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
moDC	monocyte-derived dendritic cell
MOI	multiplicity of infection
MR	mannose receptor (CD206)
Mtb	*M. tuberculosis*
MyD88	myeloid differentiation primary-response gene 88
NALP	NACHT-, LRR- and pyrin-domain-containing protein
NF-κB	nuclear factor-κB
NK	natural killer
NOD	nucleotide oligomerization domain
PAMP	Pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PD-L1	programmed cell death 1 ligand 1 (CD274)
pDC	plasmacytoid dendritic cell
PGN	peptidoglycan
PI3K	phosphatidylinositol 3-kinase
PIM	phosphatidylinositol mannosides
PRR	pattern recognition receptors
R5	CCR5 tropic
RA	retinoic acid
RICK	receptor-interacting serine/threonine kinase
RIG-I	retinoic acid-inducible gene I
RORγT	RAR-related orphan receptor γT
SIV	simian immunodeficiency virus
ss	single-stranded
STING	stimulator of interferon genes

SYK	spleen tyrosine kinase
T-bet	T-box transcription factor TBX21
TB	tuberculosis
TBK1	TANK-binding kinase 1
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper 1
TIRAP	Toll/IL-1R (TIR)-domain-containing adaptor protein
TLE	transducin-like enhancer of split
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor.
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TRIF	TIR-domain-containing adaptor protein inducing IFNβ
X4	CXCR4 tropic
XDR	extensively drug resistant

1 Introduction

1.1 The latency-instigating pathogens: *M. tuberculosis* and HIV

Latent infections are a major threat to the global human health. The two most notorious pathogens causing latent infections are the human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (Mtb). HIV-1 is the causative agent for the disease called acquired immunodeficiency syndrome (AIDS) and Mtb is the causative agent for the disease tuberculosis (TB). Approximately 34 million people are currently infected with HIV-1 and around one third of the world's population is infected with Mtb.[1,2] Each year 1.5-2 million people die from each of these infections (Fig. 1.1). The combined infection with both these pathogens is especially dangerous, as HIV-1 thrives of the immune cells that help inhibiting active TB. People having both infections are 21-34 times more likely to develop TB compared to people who are HIV-1-negative.[3] Some treatments for controlling development of AIDS and TB already exist. There is currently no vaccine against HIV-infection, however, once infected there are drugs capable of increasing HIV-positive patients' life expectancy by on average 13 years.[4] These drugs are called highly active antiretroviral therapy (HAART) and consist of three or more different drugs targeting reverse transcription and protease activity.[5] In this sense, HAART does not affect previously infected cells, but can effectively inhibit productive infection of new cells. In contrast to HIV-1, a vaccine against Mtb infection does exist. The Bacille Calmette-Guérin (BCG) vaccine was discovered in 1921 and consists of an attenuated bovine tuberculosis strain.[6] The problem with this vaccine is that it does not consistently prevent Mtb infection. In some trials it works (80% protection, Haiti trial, n = 3,100[7]) and in others it does not work at all (0% protection, India trial, n = 350,000[8]). If a person has become infected with a traditional non-drug-resistant Mtb strain, multidrug therapy for 9 months can clear the infection.[9] The eradication of one Mtb infection, however, does not protect against re-infection, and multidrug resistant (MDR) and extensively drug resistant (XDR) strains have also developed. MDR requires two years of antibiotic treatment and XDR is often untreatable and fatal.[10] Consequently, sufficient treatments against HIV-1 and Mtb do not exist, and the need for new treatment strategies is in high demand.

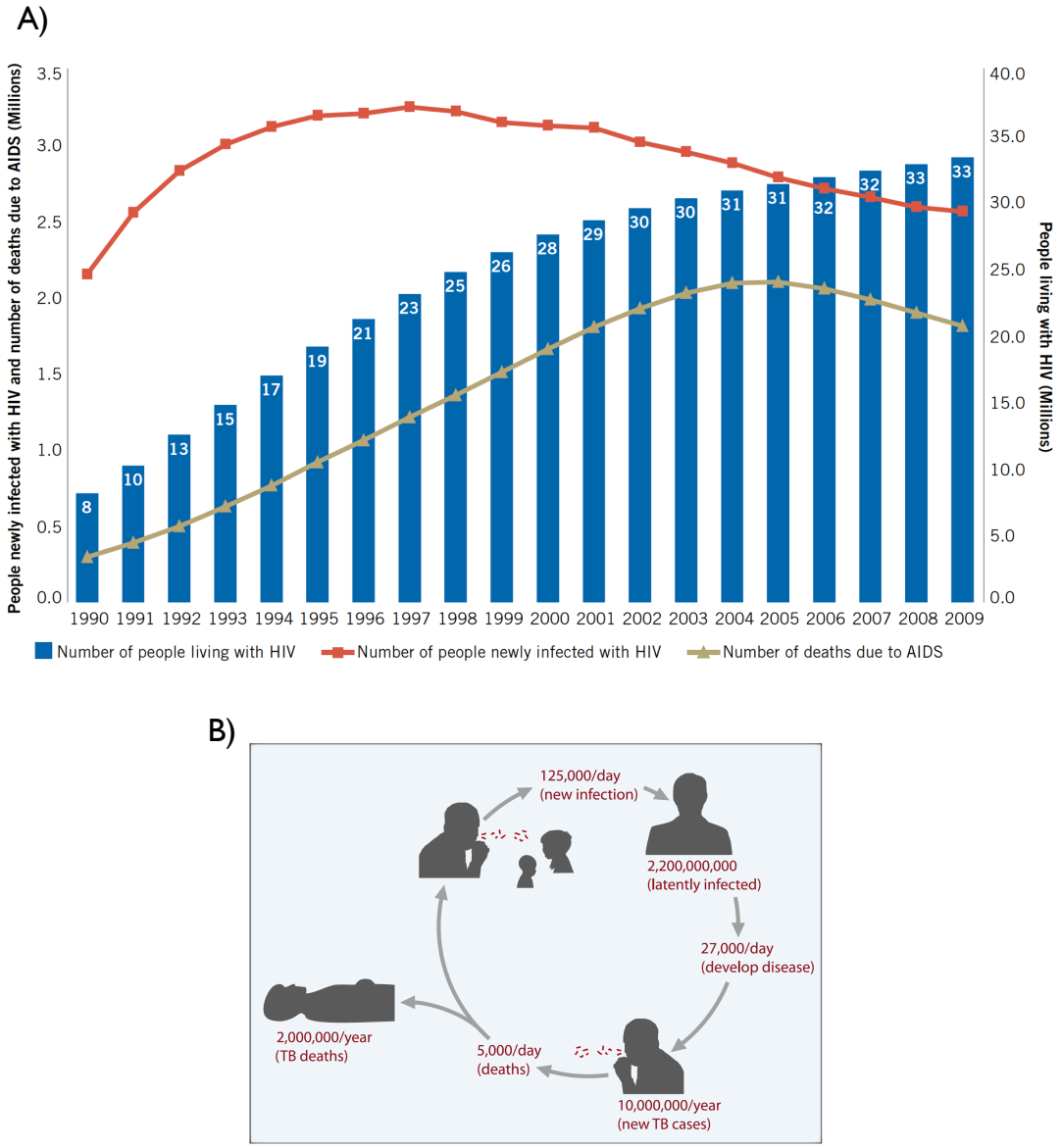

Fig. 1.1: Number of annual deaths caused by the acquired immunodeficiency syndrome and tuberculosis. A) The number of annual deaths caused by acquired immunodeficiency syndrome (AIDS) (brown line), number of people living with human immunodeficiency virus (HIV)-1 (blue bars), and number of people newly infected with HIV-1 (red line). B) Number of *M. tuberculosis* (Mtb)-infected people and annual deaths caused by tuberculosis (TB).[11–13]

Common for HIV-1 and Mtb is an exploitation of the immune system for their survival. They both mount an active immune response, but somehow the induced immune response is not sufficient, indicating that they both harbor immune-diverting abilities. In a manner similar to the Butterfly Effect,[14] the early events upon infection play an utmost important role for the progression of the immune response.[15,16] At the earliest

stage of immune activation dendritic cells (DCs) play a pivotal part. DCs are a heterogeneous population of cells, with the common attribute to initiate and regulate immune responses. They recognize when an infection has occurred, and subsequently convey the information to other immune cells including, but not limited to, naïve T cells.[17] Naïve T cells are pluripotent and can differentiate along different pathways to become distinct effector cells, depending on the additional signals received during activation. Therefore, dependent on the signals provided by the DCs, the immune system can react in many different ways. Pathogen-associated molecular patterns (PAMPs) are the basis for recognition of foreign intruders. PAMPs are conserved structures that do not resemble other structures found in the human host, and are typically essential components of microbial structure. The receptors on human immune cells that recognize these conserved patterns are called pattern recognition receptors (PRRs). Pathogens often uses mutations to avoid immune recognition, but the ingeniousness behind PRRs is that they recognize patterns that cannot be mutated, as they are essential for the pathogenicity or survival of the microorganisms. There are a range of PRR categories, with the most prominent being the toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, and C-type lectin receptors (CLRs) (Fig. 1.2). Triggering of these receptors induces activation of transcription factors including nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs) eventually leading to release of cytokines, chemokines and interferons (IFNs) and up-regulation of accessory molecules required for efficient stimulation of T cell responses necessary for pathogen clearance (Fig. 1.2).

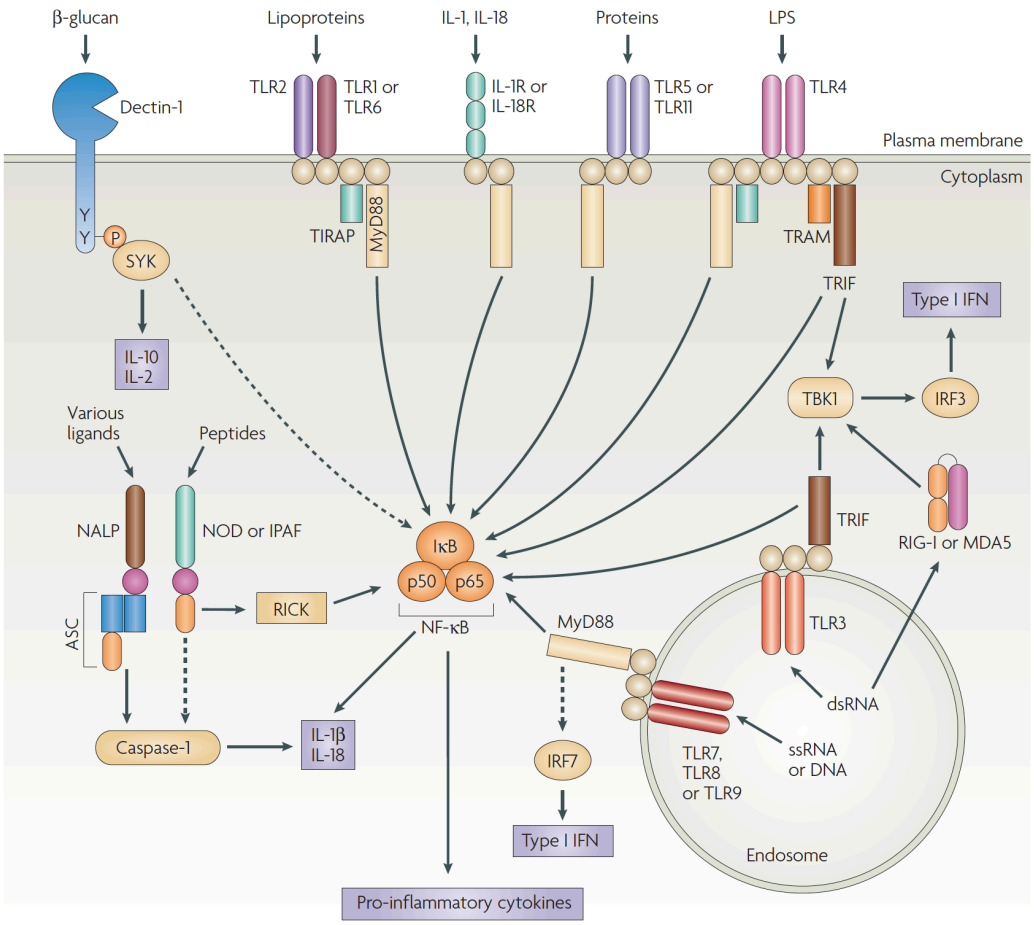

Fig. 1.2: Overview of pathogen recognizing receptors. Toll like receptors (TLRs) are found both as membrane-bound extracellular receptors (TLR1, 2, 4-6), and as endosomal receptors (TLR3, 7-9). The retinoic acid-inducible gene I (RIG-I)-like receptors: RIG-I and MDA5 (melanoma-differentiation-associated gene 5), and the nucleotide oligomerization domain (NOD)-like receptors: NOD1/2 and NALP (NACHT-, LRR- and pyrin-domain-containing protein) are all cytosolic receptors. C-type lectin receptors like Dectin-1 are membrane-bound. Dependent on cellular location and affinity for different structures, these different pattern recognizing receptors (PRRs) recognize different pathogen associated molecular patterns (PAMPs) such as lipoproteins, lipopolysaccharide (LPS), single-stranded (ss)RNA, double-stranded (ds)RNA, DNA, and glycans. Ligation induces a complex series of signaling, and only specific key molecules like nuclear factor κB (NF-κB) is shown on the figure. ASC, apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain); IFN, interferon; IκB, inhibitor of NF-κB; IL, interleukin; IPAF, ICE-protease-activating factor; IRF, IFN-regulatory factor; MyD88, myeloid differentiation primary-response gene 88; RICK, receptor-interacting serine/threonine kinase; TBK1, TANK-binding kinase 1; TIRAP, Toll/IL-1R (TIR)-domain-containing adaptor protein; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing IFN-β; SYK, spleen tyrosine kinase.[18]

Accumulating evidence suggests that HIV-1 and Mtb, harbor the capacity to escape immune recognition by circumventing the positive signals induced by PRR recognition due to negative regulation of TLR signaling.[19–26] Concurrent with TLR-binding of certain PAMPs, other ligands from the same microbe interact with regulatory receptors leading to polarization of the immune response towards tolerance. The ligands being able to induce this negative cross-regulation are primarily glycan-moieties displayed on the surface of pathogenic microbes interacting with CLRs. In this way CLRs play a dual role in the immune system, both being important in antigen uptake and at the same time being able to induce an immunomodulatory effect. However, not only CLR-triggering could divert the immune response away from a productive response. Triggering of some TLRs may inhibit pathways induced by other TLRs,[27,28] adding to the complexity of the pathogen-induced immune regulation.

Because of a different PAMP expression and localization of the infections, the specific PRRs, and cell types recognizing HIV-1 and Mtb are different. Therefore the description of interaction between these pathogens and the immune system has been divided into two different sections. Chapter 1.2-1.5 describes the immune recognition of Mtb, while chapter 1.8-1.11 describes the recognition of HIV-1. Chapters 1.7 and 1.12 describe the motivation for the studies conducted in this Ph.D. thesis.

1.2 The host response to Mtb

Mtb is spread through aerosols and enters the body through the lungs. Characteristic for the immune response against Mtb is the development of a granuloma in the lungs, consisting of an aggregate of various lymphocytes (Fig. 1.3). This limits bacterial spread, but also limits interaction of immune cells with infected cells after the granuloma has formed. Active disease evolves when bacteria spread to other sites of the body. This can for instance happen when the immune system is dampened, e.g. by virtue of an HIV-1 infection mediating a cytopathic effect on CD4+ T cells,[29–32] or when patients receive immune dampening medicine like anti-tumor necrosis factor (TNF)-α to treat rheumatoid arthritis and Crohn's disease.[33]

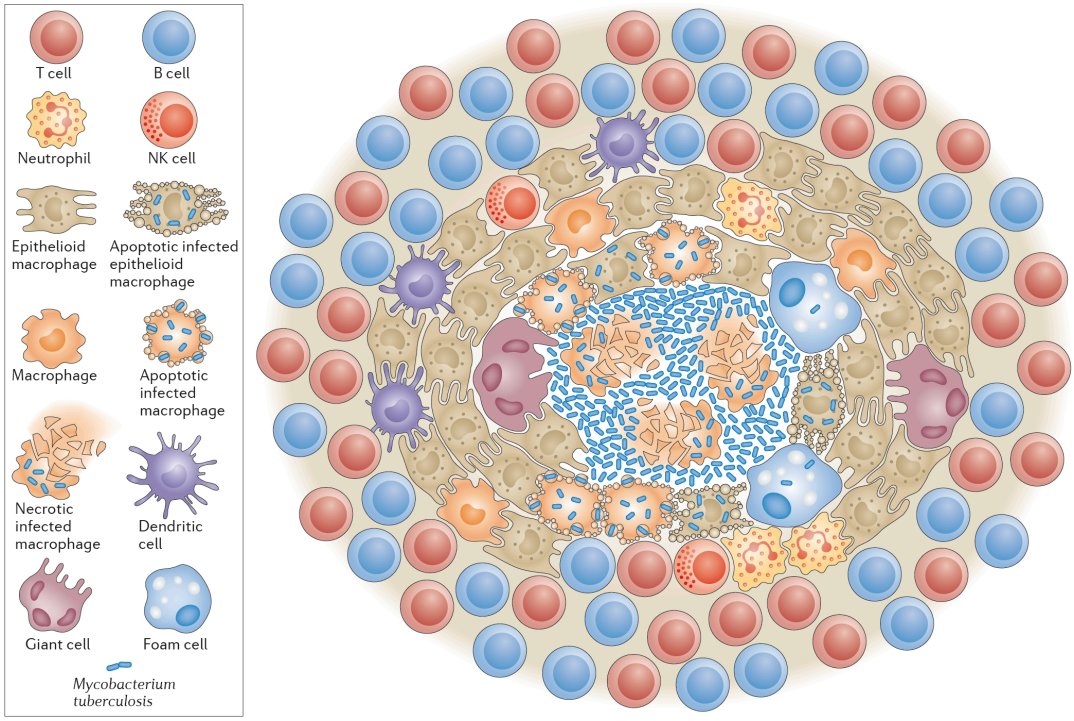

Fig. 1.3: Schematic representation of the granuloma. A granuloma is an aggregate of immune cells shielding off Mtb from the rest of the body. In the middle of the granuloma is often found a caseous-like substance consisting of dead and necrotic lymphocytes. Inside the granuloma Mtb can be found both extracellularly and intracellularly in macrophages. NK, natural killer.[34]

The first cells Mtb interacts with are the alveolar macrophages and myeloid DCs (mDCs).[35–37] Normally when microorganisms are taken up by macrophages they are degraded in the lysosomal compartment.[38,39] Mtb, however, has developed a technique to divert this by inhibiting fusion of the phagosomes with the lysosomes, and by inhibiting autophagy.[40] Unlike macrophages, Mtb is not able to grow inside mDCs.[41] Upon uptake of Mtb, mDCs migrate to the draining lymph nodes.[35,37] Here they interact with naïve T cells, presenting Mtb-derived antigen (Ag) and providing co-stimulatory and differentiation signals for the T cells.[42] After maturation the T cells migrate back to the infection site, where they help control the infection.

1.3 Interaction between Mtb and mDCs

Mtb express a range of different PAMPs that are recognized by PRRs on mDCs (Fig. 1.4). Mtb is encapsulated by a layer of peptidoglycan (PGN), which is recognized by NOD1 and NOD2 in mDCs. Inside this PGN-cell wall, lipids with varying degrees of glycosylations are embedded. Of interest to mDC recognition is mannosylated

lipoarabinomannan (ManLAM) and phosphatidylinositol mannosides (PIMs), which can be mannosylated at different degrees. The CLR DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) has a high affinity for α(1→2)-linked mannosyl residues, which are present on ManLAM and polar PIMs (like PIM6), but not apolar PIMs (like PIM1, 2 and 4).[22,43] The mannose receptor (MR) has also shown to bind ManLAM and PIM6.[43,44] PGN furthermore has lipoproteins such as LpqH, LprG, LprA, and PhoS1 embedded, which are recognized by TLR2.[45] In its core, Mtb contains genomic DNA (gDNA), which has been suggested to trigger cytosolic PRRs such as AIM2 (absent in melanoma 2) and STING (stimulator of interferon genes).[46] gDNA also binds TLR9,[47] but because of divergent reports on whether or not TLR9 is expressed in mDCs[48–50] it is difficult to say whether TLR9 is involved in Mtb-recognition in mDCs. TLR4 has also been attributed to protection against Mtb,[51,52] and has been shown to be triggered by monoacylated muramyl dipeptide (a constituent of PGN)[53] and the newly discovered Mtb-ligand heparin-binding hemagglutinin.[54]

Upon activation of mDCs by Mtb, they undergo a process of maturation. They up-regulate the antigen-presenting molecule major histocompatibility complex (MHC) class II, and the co-stimulatory molecules cluster of differentiation (CD)40, CD80 and CD86,[28,55–57] and also the inhibitory ligand programmed cell death 1 ligand 1 (PD-L1).[58] They secrete interleukin (IL)-6, IL-10, TNF-α, IL-1α, IL-1β, IL-23 and IL-12p70, but the latter is always in very small amounts.[28,55–57] Collectively, the cytokines secreted by mDCs upon interaction with Mtb are able to induce both T helper (Th)1 and Th17 responses, with the Th1 response being the most warranted for clearance of the infection.

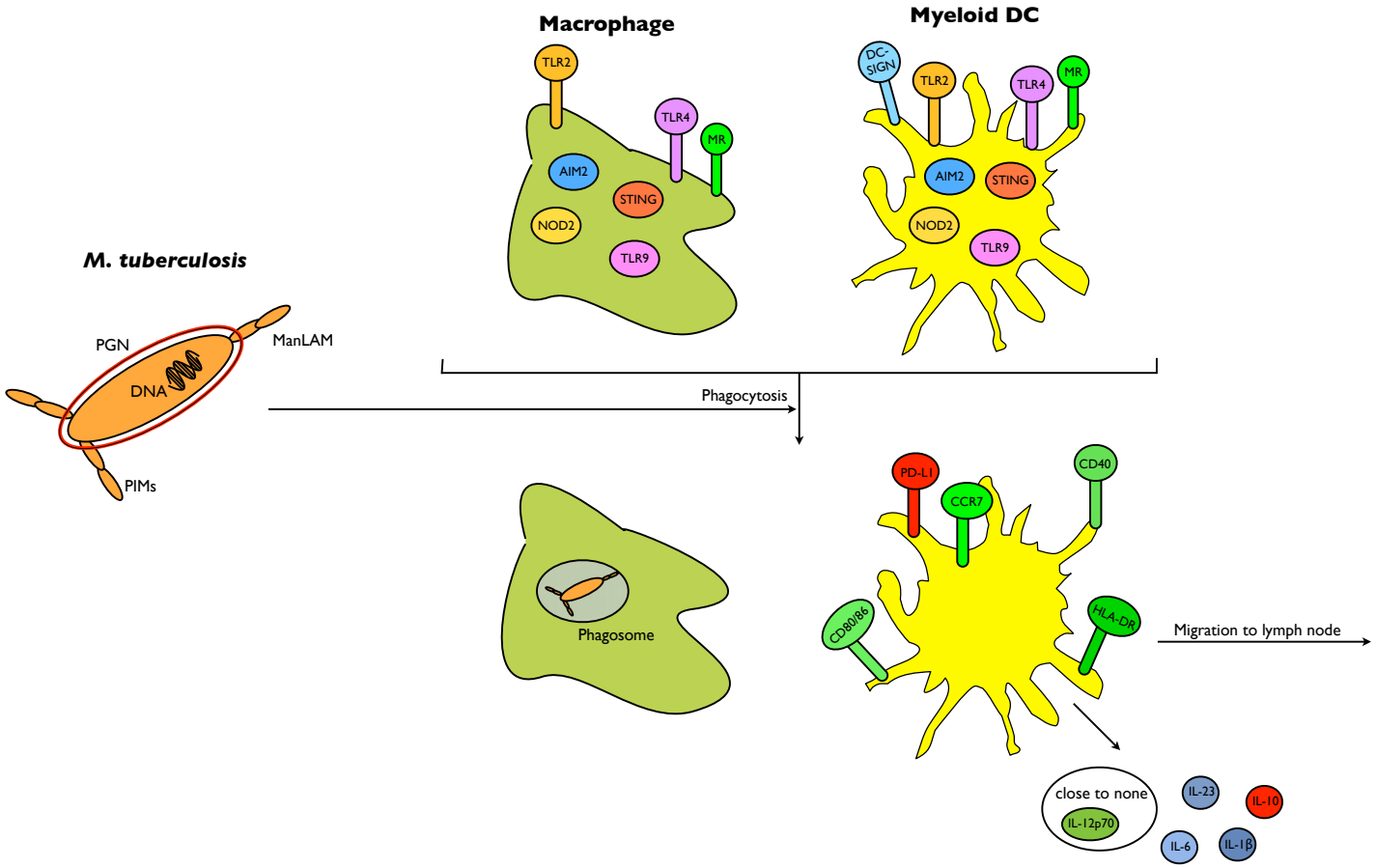

Fig. 1.4: Early interaction of Mtb with the immune system. Upon entrance of Mtb into the lungs, interaction between macrophages and myeloid dendritic cells (mDCs) occur. Mtb expresses a range of PAMPs and the immune cells expresses a range of PRRs. PAMPs and PRRs are indicated on the upper part of the figure. Mtb is not degraded in macrophages, and instead these cells become a reservoir for Mtb. mDCs on the other hand matures upon Mtb-interaction, visualized by the upregulation of surface markers, expression of cytokines and their migration to the draining lymph node. AIM2, absent in melanoma 2; CCR, C-C chemokine receptor; CD, cluster of differentiation; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; ManLAM, mannosylated lipoarabinomannan; MR, mannose receptor; PGN, peptidoglycan; PD-L1, programmed cell death 1 ligand 1; PIM, phosphatidylinositol mannoside; STING, stimulator of interferon genes.

1.4 Mtb and Th responses

The role of a Th1 response during an Mtb infection is 1) to enhance the intracellular antimicrobial defenses of macrophages,[59–62] and 2) to provide activation help for CD8+ cytotoxic T lymphocytes (CTLs) in order to kill infected macrophages.[63–67] Th1 cells activate macrophages by virtue of IFN-γ, CD40ligand (CD40L)/CD40-interactions, and T cell receptor (TCR)/MHC class II-interactions. This enables the

phagosome to fuse with the lysosome, and induces the production of reactive nitrogen and oxygen species inside the macrophage. Th1 cells provide help for CTLs by producing IL-2, and increasing co-stimulatory molecule expression on the macrophage, mediating increased MHC class I co-stimulation for the CTLs. This enables perforin/granzyme B-mediated killing of the infected macrophage. The importance of the Th1-response in keeping mycobacterial infections under control can be seen from patients with a mutation in one of the genes: *IFNGR1, IFNGR2, STAT1, IL12B,* and *IL12BR1*. Collectively these patients are diagnosed with a disease called "Mendelian susceptibility to mycobacterial disease", and as the name of the disease reflects, these patients are highly susceptible to Mtb infection.[68] Furthermore, IFN-γ knockout mice quickly succumb to Mtb infection.[69,70]

IL-12p70 is the most important mDC-produced cytokine for induction of a Th1 response.[71,72] However, IL-27 has also been linked with development of a Th1 response, e.g. by inducing naïve CD4+ T cells to upregulate IL-12Rβ2, and to express the Th1-specific transcription factor T-box transcription factor TBX21 (T-bet).[73,74] Another important Mtb-induced mDC-derived cytokine is IL-10. IL-10 has a general immune suppressive activity and can therefore both inhibit clearance of Mtb, and limit exacerbated pathologic immune activation. In experimental systems the former is often seen. For instance it has been shown that autocrine IL-10 suppresses mDC-mediated IL-12p70 production and migration of mDCs to the lymph nodes during a mycobacterial infection.[75] IL-10 knockout mice are also superior in withstanding Mtb-infection caused by a faster and enhanced Th1 response in the lungs.[76] Furthermore, IL-10 has also been shown to inhibit autophagy, that otherwise would participate in the protective immune response against Mtb.[77]

Mtb has also been shown to induce Th17-responses. For instance it has been shown that IL-17-producing long-lived central memory CD4+ T cells are found in the peripheral blood of mycobacteria-infected, otherwise healthy, human adults.[78] Whether these peripheral blood Th17 cells has any protective role in the human immune response to Mtb remains to be elucidated. In the murine system, it has been shown that Th17 cells impacts the Mtb-induced inflammation, but does not affect overall protective immunity.[79,80] Another study has shown that an Mtb-induced Th17-response may be important in accelerating a Th1-recall response by inducing production of the

chemokines chemokine (C-X-C motif) ligand (CXCL)9, CXCL10 and CXCL11 by lung mucosal epithelial cells, which in turn recruits Mtb Ag-specific chemokine (C-X-C motif) receptor (CXCR)3+ Th1 cells.[81] Overall this suggests that Th17 responses are not necessarily needed for primary protection against Mtb, but may be useful in recall responses to boost Th1 responses. The Th17 response to Mtb is initiated in the lung draining lymph node when mDCs interact with naïve CD4+ T cells and prime them to become Th17 cells. Cytokines secreted by these mDCs are pivotal for the type of Th cell induced. IL-23, IL-1β, and IL-6 have been associated with the generation of effector human Th17 cells, although the exact combination of cytokines varies between studies.[82–85] When using human thymic and umbilical cord blood naïve CD4+ T cells, the combination of IL-1β and IL-23 was especially important for Th17 induction.[86] Transforming growth factor (TGF)-β was for a while believed to inhibit Th17 induction,[83–85] but recently it was demonstrated that TGF-β plays an indirect role in development of human Th17 cells, because Th17 cells are less susceptible to suppression by TGF-β than Th1 and Th2 cells are.[87] Therefore TGF-β may play an indirect role in formation of Th17 cells by inhibiting differentiation into Th1 and Th2.

Regulatory T cells (Tregs) are also induced during an Mtb-infection,[88–90] and dampens the immune response towards Mtb. For instance it was shown that depletion of Tregs in mice leads to a decreased number of Mtb in the lung and spleen,[91] which may be caused by a Treg-mediated inhibition of effector T cell accumulation and function in the lungs, causing prolonged initial bacterial expansion.[92] As one of the major effector molecules from Tregs is IL-10, mDC-derived IL-10 could also induce a delayed immune response against Mtb. Furthermore mDC-produced TGF-β and retinoic acid (RA) can also induce the generation of Tregs.[93–96]

Fig. 1.5 summarizes the different Th responses seen during an Mtb infection, as well as their effector functions. The mDC-produced cytokines that leads to the induction of the different phenotypes are also shown on the figure.

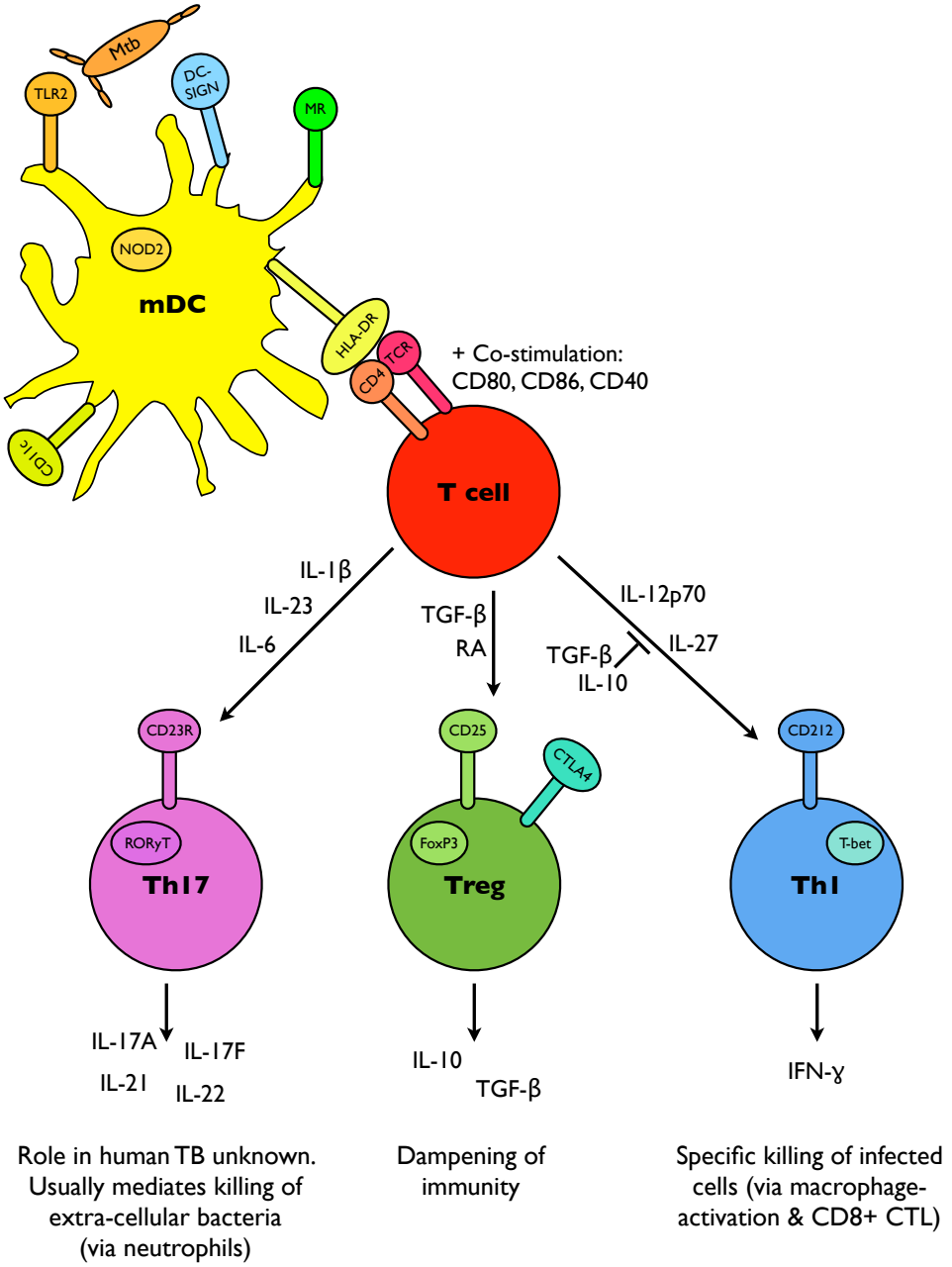

Fig. 1.5: Summary of mDC-induced T helper responses against Mtb. An Mtb infection has been associated with T helper (Th)1, Th17 and regulatory T cell (Treg) responses. In order for naïve CD4+ T cells to differentiate into these specific subsets, instructions must be received from antigen-presenting mDCs in the lymph nodes. Dependent on the PAMPs recognized by the mDCs different responses are initiated, demonstrated by different cytokine response patterns. The different DC-produced cytokines linked with the differentiation into the T cell subsets are indicated on the figure. The shown cytokines have not all been confirmed to be induced in mDCs after Mtb interaction, but their role in Th differentiation has been determined elsewhere. The effector mechanism of the different Th cell subsets is described in the bottom of the figure. Phenotypic markers for Th cell subsets displayed on this figure: CD23R is the IL-23 receptor, CD212 is the IL-12p70 receptor (β1-subunit), CD25 is the IL-2 receptor (α-chain), and CTLA-4 (cytotoxic T-lymphocyte antigen-4) is a co-

inhibitory receptor. Transcription factors specific for each Th cell subset are shown inside each Th subset. CTL, cytotoxic T-lymphocyte; FoxP3, forkhead box P3; HLA-DR, human leukocyte antigen-DR; RA, retinoic acid; RORγT, RAR-related orphan receptor γT; T-bet, T-box transcription factor TBX21; TCR, T cell receptor; TGF, transforming growth factor.

1.5 Regulation of Th-inducing cytokines in mDCs

Currently the conjecture is that Mtb induces both Th1 and Th17 responses, often in literature described as a Th1/Th17 response. However, reports have indicated that IL-12p70 and IL-23 may be reciprocally regulated.[27,28] Given the essentiality of IL-12p70 and IL-23 in inducing human Th1 and Th17 cells respectively, the regulation of these cytokines within mDCs may play a central role for the initial immune response against Mtb. Of the PRRs found in mDCs capable of recognizing Mtb, it has been shown that TLR4 and TLR9 signals through p38 and c-Jun N-terminal kinase (JNK)1/2 to induce IL-12p70 (Fig. 1.6).[97–99] TLR2 and DC-SIGN have been shown to signal through extracellular-signal-regulated kinase (ERK)1/2 to induce IL-23, IL-1β and IL-10.[23,100,101] DC-SIGN signaling furthermore include signaling through Raf-1.[101] ERK1/2 signaling and the transcription factor c-Fos has furthermore been shown to inhibit IL-12p70 production,[97,99,102,103] although it is currently not known whether the inhibition is an indirect effect of an enhanced IL-10 production. Finally, triggering of MR induces signaling through IL-1 receptor-associated kinase 3 (IRAK-M),[104] which blocks TLR signaling.[105] A summary of these signaling pathways induced by Mtb can be seen on Fig. 1.6.

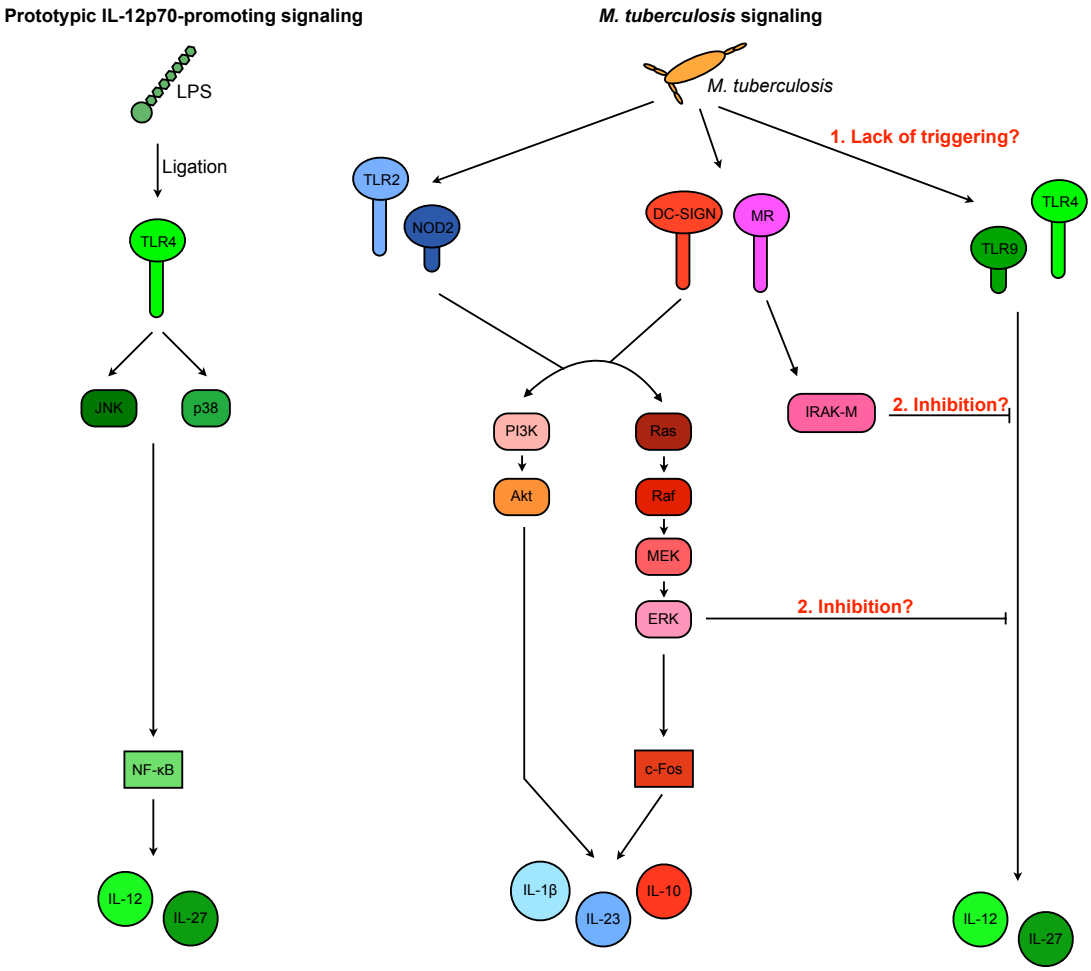

Fig. 1.6: Intracellular signaling pathways regulating cytokine production in human myeloid dendritic cells. The optimal immune response Mtb would include a Th1 response, and signaling that induces this type of response can be visualized by the prototypic IL-12p70-inducing stimuli LPS. Only little IL-12p70 production has been noticed after interaction between mDCs and Mtb, but whether this is due to a lack of triggering of the receptors inducing this response or an active inhibition of the response is not known. It has been demonstrated that Mtb triggers the extracellular-signal-regulated kinase (ERK) pathway, which has been linked with production of IL-1β, IL-23 and IL-10 and inhibition of IL-12p70 production, thus suggesting that active inhibition could take place. Green: Th1, blue: Th17, red: immune dampening. IRAK-M, IL-1 receptor-associated kinase 3; JNK, c-Jun N-terminal kinase; MEK, mitogen activated protein kinase/ERK kinase; PI3K, Phosphatidylinositol 3' kinase.

Of major importance to the development of the immune response against Mtb, is a very low, almost neglectable, production of IL-12p70 by human mDCs.[28,55–57] Two different schemas could explain the diminished Mtb-induced IL-12p70 response in mDCs: 1) There is too little/no triggering of IL-12p70-promoting TLRs in mDCs after interaction with Mtb, and/or 2) Mtb triggers pathways that actively downregulate IL-

12p70 induction. Experiments have shown that blocking the ERK or phosphatidylinositol 3-kinase (PI3K) pathways in monocyte-derived macrophages increases IL-12p70 production after Mtb-triggering,[106] thus suggesting that the latter scenario could be in play. Further evidence for mechanisms that actively repress IL-12p70 production has recently been published.[27] It was shown that the TLR2/CLR ligand zymosan activates transcriptional repressors (hairy and enchancer of split-1 (Hes)1, Hes5, hairy/enhancer-of-split related with YRPW motif protein (Hey)1 and transducin-like enhancer of split (TLE)) of *IL12A* (encoding IL-12p35 subunit of IL-12p70) leading to c-Rel dissociation from the *IL12A* promoter. LPS on the other hand did not induce these repressors, enabling c-Rel to stay on the *il12a* promoter region for a longer period of time, leading to transcription of IL-12p35, and production of bioactive IL-12p70 protein.

1.6 Monocyte-derived DCs as an experimental system

Much of what we know about the interaction between Mtb and mDCs originate from research conducted on an *in vitro* analogue to the mDCs found *in vivo*. These *in vitro* generated mDCs are called monocyte-derived DCs (moDCs), because they are differentiated from monocytes in culture using a combination of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF).[107] Blood-derived mDCs and moDCs share many of the same properties such as TLR-expression-repertoire and the cytokines secreted in response to TLR-ligation.[108] The main advantage of using moDCs is the ability to generate large amounts of cells to study (typically 3-6*10^7/donor after differentiation). Blood-derived mDCs are very scarce, typically constituting 0.3-0.9% of peripheral blood mononuclear cells (PBMCs),[109,110] and giving a yield of 2-5*10^5/donor after purification. Lung-resident mDCs are even harder to study, as acquisition of these requires an invasive biopsy, and can only be acquired from surgeries of therapeutic or diagnostic purposes such as thoracotomies, lobectomia or pneumectomia.[111,112] Ten Berge *et al.*[113] furthermore argues against the use of such tissue, as the diseased tissue, and the subsequent enzymatic tissue-digestion step may have influenced the phenotype and function of the mDCs. Instead Ten Berge *et al.* suggest using mDCs from bronchoalveolar lavage fluid (BALF). However, like the blood-derived mDCs, the numbers of mDCs in BALF are very scarce, and typically only 5-10*10^4 mDCs/donor can be acquired prior to any purification steps.[114] By vir-

tue of the large amount of moDCs that can be generated, advanced studies such as kinome-wide phosphoproteomics can be conducted. It has furthermore been demonstrated that moDCs migrate into the lungs of mice,[115] suggesting a direct *in vivo* relevance of moDCs in Mtb-infection. Confirming the results obtained from moDC studies in lung-resident mDCs is desirable, but outside the scope of this thesis.

1.7 Motivation for paper 1-3

The motivation behind three of the papers included in this thesis has been to determine how Mtb evades propagation of protective immunity by subverting the phenotype of human moDCs. Paper 1 focuses on the plasticity of moDCs, and demonstrates a donor-dependent ability of monocytes to differentiate into two distinct moDC subtypes, with only one of them being capable of producing Th1-propagating IL-12p70. Paper 1 furthermore demonstrates how Mtb exploits plasticity in the IL-12p70-competent moDCs to induce a subset of cells that lacks IL-12p70-competence. In paper 2, a method for isolation of the IL-12p70-competent moDCs is described, and paper 3 focuses on the use of these purified IL-12p70-competent moDCs to study the signaling-transduction networks induced by Mtb in moDCs. The phenotype of naïve CD4+CD45RA+ T cells co-cultured with Mtb-primed moDCs was also examined, and in paper 3 it is demonstrated how Mtb skews the Th response towards Th17. The outcome of these studies can be found in chapters 2, 3 and 4.

1.8 The host immune response against HIV-1

HIV-1 does not kill a person by means of cytotoxic attacks like other lethal microorganisms. Instead it eliminates the patient's Th cell-based immune system, leaving the body unprotected from other infections. This eradication of the immune system leads to the disease called AIDS. In recent years much focus has been given to the early events after HIV-1 enters the body, and the initial period before peak viraemia 21-28 days after infection has been called the window of opportunity for eradication of the infection.[15,116] Therefore understanding the initial interaction between HIV-1 and the immune system could lead to new treatment strategies for this deadly infection.

HIV-1 enters the body at vaginal and rectal mucosal surfaces.[15] Here they interact with cells from the immune system expressing the surface molecule CD4. This includes T cells, macrophages and different DCs (Fig. 1.7 and 1.8). The HIV-1 envelope glycoprotein (gp)120 binds CD4 on human immune cells in combination with one of the two co-receptors C-C chemokine receptor (CCR)5 and C-X-C chemokine receptor (CXCR)4.[117–119] Initially gp120 binds CD4, which induces conformational changes in gp120 that enables binding to a co-receptor.[120] This triggers a second conformational change that allows insertion of a fusion peptide into the host cell membrane.[121,122] This, in turn, enables HIV-1 to enter the host cell and integrate into the host genome, thereby resulting in viral replication (Fig. 1.7).

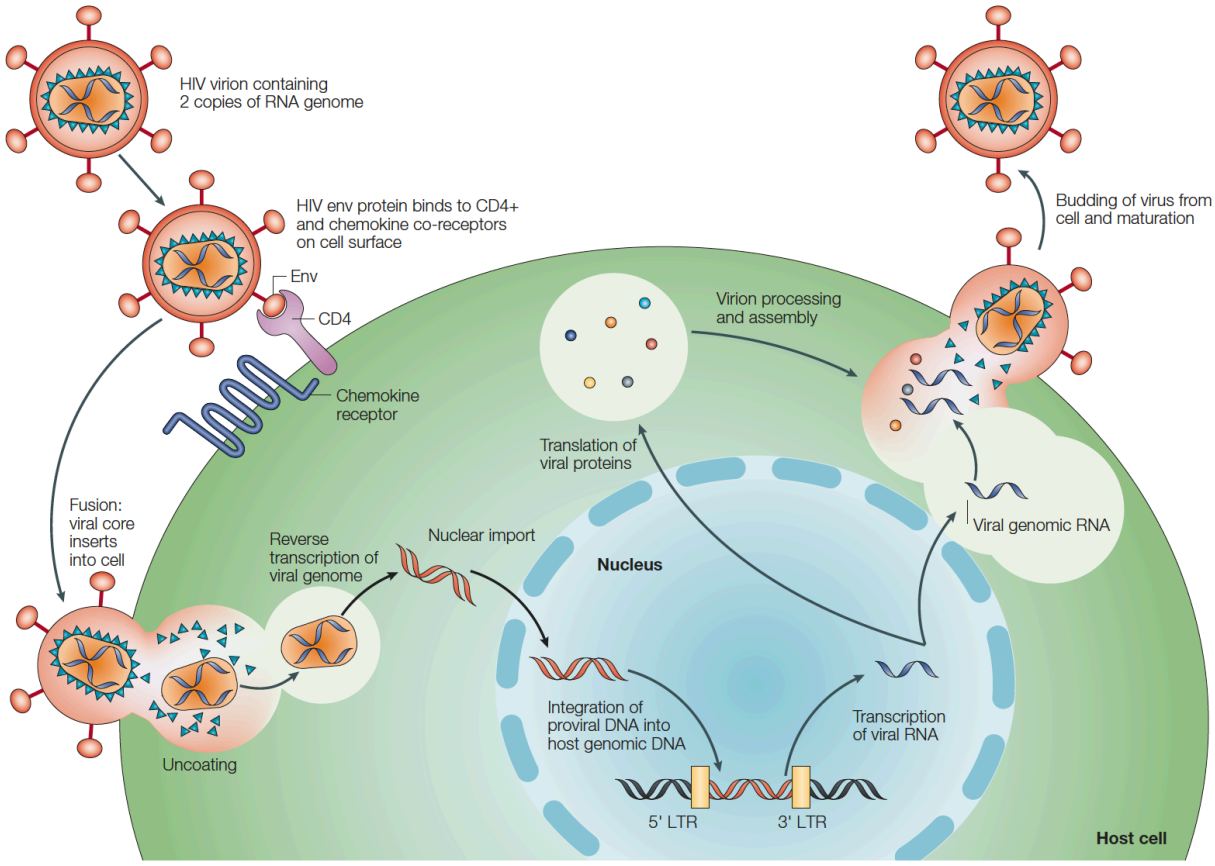

Fig. 1.7: Schematic representation of the HIV-1 life cycle. HIV-1 enters immune cells upon interaction with CD4 and one of the chemokine receptors CCR5 and chemokine (C-X-C motif) receptor (CXCR)4. Inside the cell HIV's RNA genome is reverse transcribed into DNA, which subsequently is integrated into the host genome. Consequently, HIV-1 can use the host cell's own machinery to replicate, when the cell needs to transcribe the genes that HIV-1 has integrated into. NF-κB is among the transcription factors shown to induce viral replication. The *de novo* generated viral RNA encodes proteins needed for the viral capsid and assembly thereof. When new viral particles have assembled, HIV-

1 buds from the host cell and is subsequently capable of infecting new cells. Env, gp120; LTR, long terminal repeat.[123]

On the basis of the two types of co-receptors, different HIV-1 strains have been divided into two tropisms, namely CCR5 tropic (R5) and CXCR4 tropic (X4) HIV-1 strains. Individuals infected with HIV-1 are exposed to many different HIV-1 strains at the mucosal surface, but only few HIV-1 strains are able to establish infection.[116,124,125] Recent data has indicated that in 70-80% of cases the founder population originates from a single virion.[116] In far the most cases, this strain is R5 and not X4,[124,125] but currently the reason for this tropism-selectivity is not fully understood. The major targets for X4 HIV-1 strains are naïve CD4+ T cells and thymic precursors,[126,127] while the major targets for R5 HIV-1 strains are effector T cells and macrophages.[127–129] Infection of effector memory T cells present at the mucosal surfaces, leads to viral replication and apoptosis of the T cells (Fig. 1.8).[130–132] Macrophages on the other hand are more resistant to the cytopathic effect of HIV-1,[133,134] and therefore acts as a long-term reservoir for HIV-1 (Fig. 1.8).[129] Macrophages have furthermore been shown to be involved in transferring HIV-1 to T cells,[135,136] but whether they play any protective role during an HIV-1 infection is not known.

1.9 DCs and HIV-1

Different types of DCs are also present at the mucosal surfaces. In humans it has been shown that CD11c+DC-SIGN+MHC class II+ mDCs are present in the lamina propria and the interfollicular regions of Peyer's patches, and IL-3R+MHC class II+ plasmacytoid DCs (pDCs) are present in the high endothelial venules and the interfollicular regions of Peyer's patches.[137] Furthermore pDCs arrive at the site of simian immunodeficiency virus (SIV)-infection in rhesus macaques as early as one day after infection,[138] indicating that they could play an important role in the early phase of the immune response against HIV-1. Both mDCs and pDCs express CCR5 and CXCR4 and are therefore capable of interacting with HIV-1 strains from both tropisms (Fig. 1.8). As described earlier, DCs play a major part in initiating the adaptive immune response and are therefore very important in the anti-viral immune response. Usually when DCs encounter an intruding pathogen they advance from an immature state to

an activated state characterized by the upregulation and secretion of several different immune regulatory proteins. Notably, mDCs are not activated by HIV-1 even though they express TLR8 capable of recognizing ssRNA (Fig. 1.8).[139,140] pDCs on the other hand do get activated by HIV-1, and upon interaction, pDCs produce type I IFNs, up-regulate antigen-presenting, co-stimulatory and inhibitory molecules, and gain migratory capacity towards chemokine (C-C motif) ligand (CCL)19 by upregulation of CCR7[140–143] (Fig. 1.8).

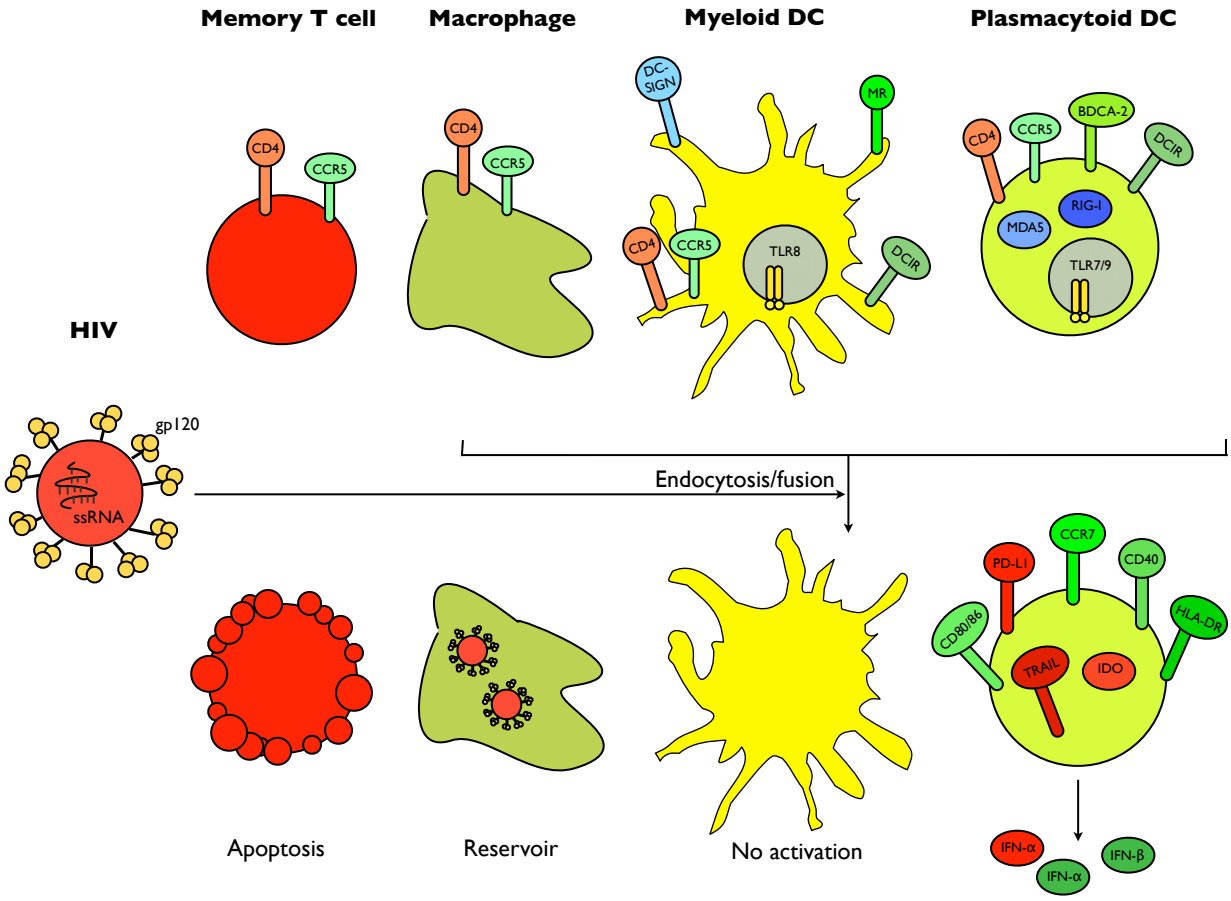

Fig. 1.8: Proposed initial interactions between HIV-1 and the immune system. HIV-1 enters the body at mucosal surfaces where interaction between the virus and immune cells expressing CD4 and CCR5 is taking place. The upper panel shows receptors on the immune cells capable of interacting with HIV-1. The lower panel shows the effect of interaction with HIV-1 on the immune cell phenotype. Upon interaction with HIV-1, memory T cells and macrophages are infected and either undergo apoptosis or act as a reservoir for HIV-1 replication. mDCs are not infected or activated by HIV-1. Plasmacytoid DCs (pDCs) are activated leading to secretion of type I IFNs and upregulation of a range of surface markers. BDCA, blood dendritic cell antigen; DCIR, dendritic cell immunoreceptor; IDO, indoleamine 2,3-dioxygenase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

1.10 Recognition of HIV-1 by pDCs

pDCs express a number of receptors capable of recognizing various PAMPs from HIV-1.[144] In the endosomes, TLR7 and TLR9 are localized and recognize single-stranded (ss)RNA and double-stranded (ds)DNA/unmethylated CpG sequences, respectively. In the cytosolic compartment RIG-I recognizes 5' triphosphorylated ssRNAs and relatively short dsRNA, and melanoma-differentiation-associated gene 5 (MDA5) recognizes long dsRNAs. Curiously, DNA retrotranscripts do not activate pDCs.[141] One reason for the inactivity of HIV-1 DNA is the low frequency of CpG motifs when compared to bacterial and other viral DNAs.[145] Recently HIV-1 has been shown to be recognized by RIG-I in PBMCs and monocyte-derived macrophages,[146] but these findings have not yet been replicated in pDCs. It is therefore currently believed that the main PRR in pDCs for HIV-1 recognition is TLR7.[141] Besides ssRNA, HIV-1 also carries a CLR-triggering ligand, namely high-mannose structures present on gp120. These high-mannose structures have been shown to interact with a range of receptors both on pDCs and mDCs. On pDCs, blood dendritic cell antigen (BDCA)-2[21] and dendritic cell immunoreceptor (DCIR)[19,147] are able to bind gp120. mDCs also express DCIR, and furthermore express MR and DC-SIGN. Gp120-ligation to CLRs has been shown to modulate TLR-triggered signaling in both DC subtypes,[147–149] and it has been suggested that the lack of HIV-1-mediated activation of mDCs could be promoted by high-mannose-CLR-signaling.

1.11 Effector mechanisms of pDC activation

Probably the most important effect of pDC activation by HIV-1 is a very rapid production of type I IFNs. pDCs are especially fast at inducing IFN-α compared to other immune cells, as pDCs constitutively express IRF7 necessary for IFN-α induction.[150] IFN-α has a direct killing effect on HIV-1, but also plays a role in the activation of other immune cells (Fig. 1.9). It has been shown that mDCs can be activated during an HIV-1 infection by virtue of pDC-derived type I IFNs.[140] pDC-derived type I IFN also activates natural killer (NK) cells,[151] and has in general been shown to induce Th1 polarization as evidenced by T-bet expression.[152] pDC-derived IFN-α furthermore limits HIV-1 replication in CD4+ T cells,[153,154] but prolonged IFN-α production

can also lead to T cell death by TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis.[155,156] Hence, type I IFNs can both have beneficial and detrimental effects on the immune response against HIV-1. Another detrimental effect of HIV-activation of pDCs is the upregulation of indoleamine 2,3-dioxygenase (IDO), which in turn induces naïve CD4+ T cells to differentiate into Tregs.[157,158] These Tregs suppress T cell proliferation and mDC maturation. As for the dichotomy seen for IFN-α, it is currently not known whether IDO-expression has a beneficial effect by decreasing the immunopathology or a detrimental effect by inhibiting proper viral clearance.

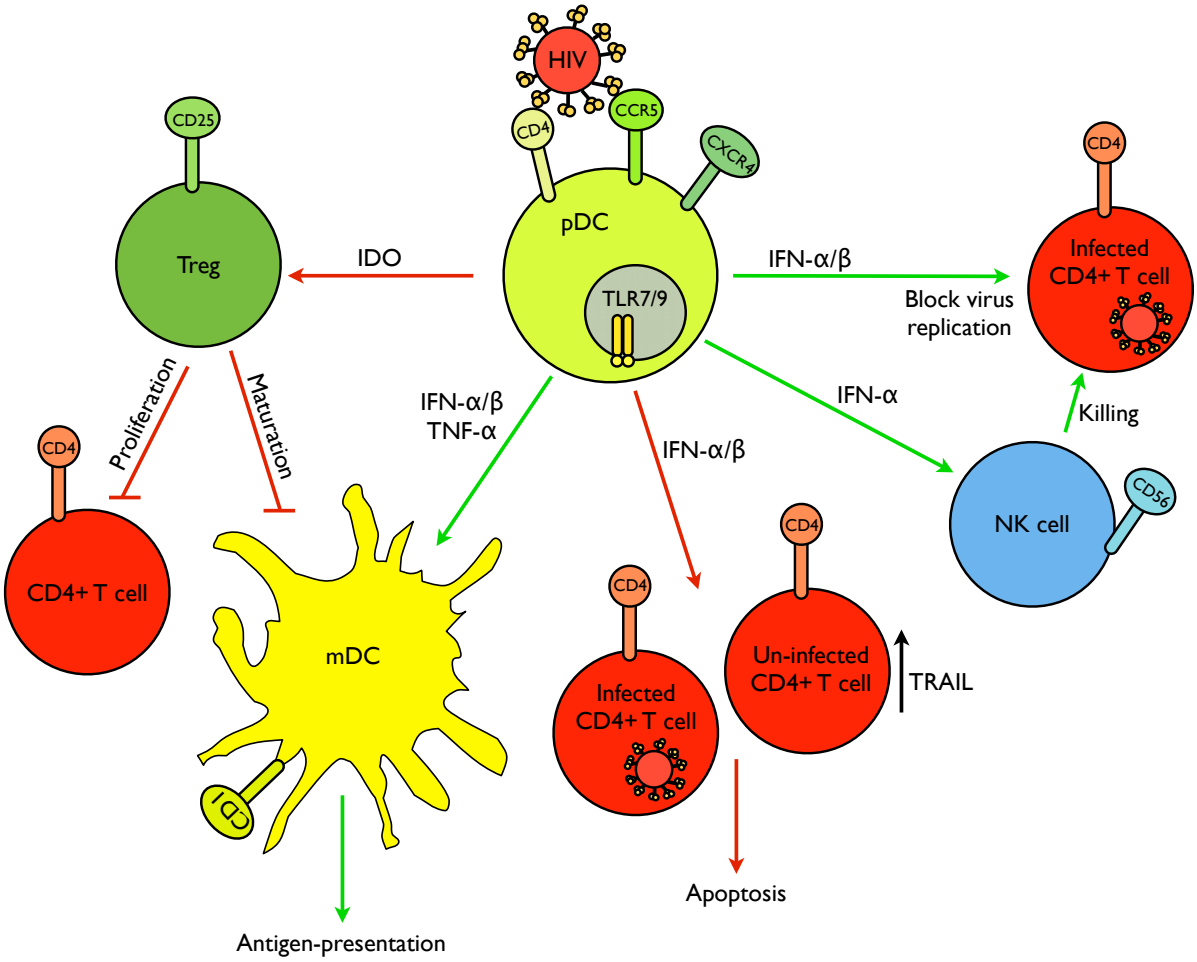

Fig. 1.9: Effect of HIV-1-mediated pDC-activation on other immune cells. The main effector molecules induced after activation of pDCs by HIV-1 is type I IFNs. These have both beneficial (green lines) and detrimental (red lines) effect on the immune response towards HIV-1. pDCs also upregulate IDO, which has an immune dampening effect. CD56 is a NK cell specific marker. TNF, tumor necrosis factor.

1.12 Motivation for paper 4

Studies on CLR-TLR crosstalk in pDCs has been conducted using recombinant gp120, or Ab-triggering of receptors. Recently it was demonstrated how glycosylation patterns on recombinant gp120 is affected by the expression system used to produce the protein, and may therefore not reflect natural HIV-1 glycosylation.[159] Consequently, it is currently not known if HIV-1 natural gp120 glycosylation will affect the phenotype of pDCs. Given the very important role pDCs plays in the anti-viral immune response, gp120-mediated dampening of pDC activity could have dire consequences for the immune response against HIV-1. Therefore, paper 4 focused on the interaction between pDCs and HIV-1, and elucidation of the role played by high-mannose structures for immune evasion (Fig. 1.10). This paper can be found in chapter 5.

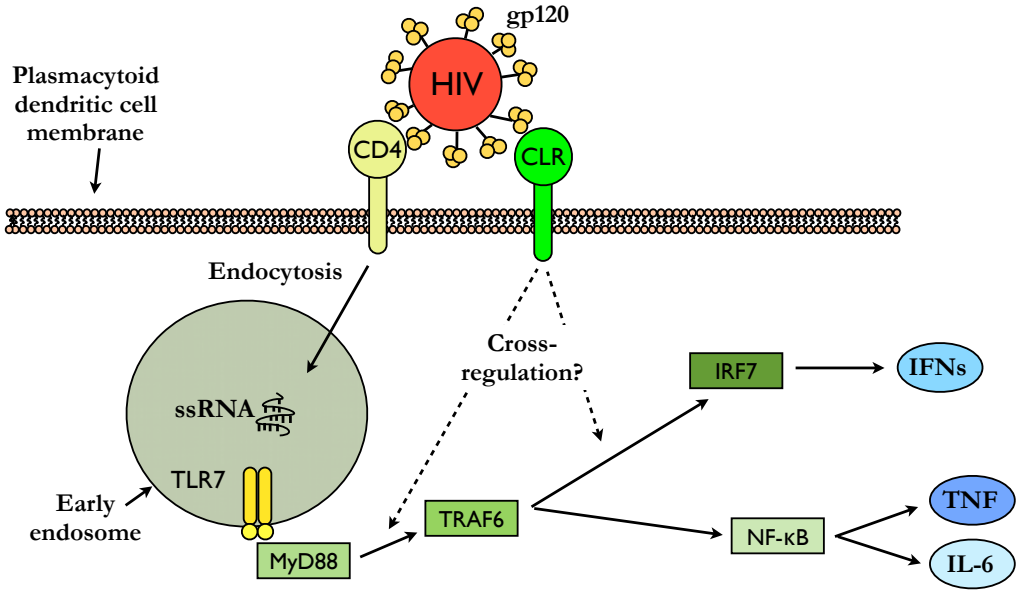

Fig. 1.10: Potential cross-regulation between TLR7 and CLRs in pDCs. pDCs mainly recognize HIV-1 by virtue of TLR7, but CLRs have also been shown to bind HIV-1 glycoprotein (gp)120. The cross-regulation between these receptors has not been thoroughly investigated and has therefore been the focus of paper 4 in this thesis. TRAF, TNF receptor associated factor.

2 *M. tuberculosis* regulates dendritic cell plasticity towards high CD14 and low IL-12p70 that reprograms upon exogenous IFN-γ

Authors

Jonas N. Søndergaard & Susanne Brix

2.1 Abstract

Immunity against *Mycobacterium tuberculosis* (Mtb) depends on the way dendritic cells (DCs) interpret the bacterial constituent matters. Given the intracellular character of Mtb, a preferable DC signature is based particularly on IL-12p70 to propagate IFN-γ-producing CD4+ T-cells. Therefore it is perplexing that IL-12p70 levels are weak in monocyte-derived DCs (moDCs) in response to Mtb. Here we report that Mtb modifies moDC plasticity by expanding a CD14-high expressing subset with weak IL-12p70-producing capacity. CD1a expression levels were found to divide moDCs into two subsets at the immature state, while enhanced CD14 defined a third subset propagated after Mtb activation, but not by lipopolysaccharide (LPS). Irrespective of LPS- or Mtb-stimulation, the CD1a-high, CD14-low moDCs proved the main IL-12 family cytokine producers. The Mtb-promoted subset, characterized by CD14-high expression, exhibited similar chemokine receptor and cytokine expression patterns as CD1a-low moDCs; being weak IL-12p70 producers and poor expanders of IFN-γ in naïve CD4+ T-cells. Mtb-derived peptidoglycan and mannosylated lipoarabinomannan partly explained the subset propagation. Addition of IFN-γ, but neither IL-17A nor IL-22, inhibited the characteristic subset division and promoted high level IL-12p70 in Mtb-challenged moDCs. We conclude that Mtb exploit moDC plasticity to reduce production of IL-12p70, being entirely divertible by exogenous IFN-γ.

2.2 Introduction

A *Mycobacterium tuberculosis* (Mtb) infection originates by inhalation of the bacteria into the lungs. The first tissue-resident cells Mtb interacts with are therefore the alveolar macrophages and dendritic cells (DCs).[35–37] Mtb avoid getting killed by phagocytosing macrophages by inhibiting fusion of the Mtb-containing phagosome with the lysosome, and by inhibiting autophagy.[40,77] Consequently, Mtb obtains status as an intracellular microbe that survives within lung-resident macrophages. Clearance of this type of pathogen most essentially requires a CD4+ Th1 immune response needed to activate infected macrophages and to provide help for CD8+ cytotoxic T lymphocytes capable of killing infected cells.[59–61,66] Because DCs are the only cells capable of activating naïve T cells, the way DCs respond to Mtb becomes a critical factor for how the immune response to Mtb evolves. DC-produced IL-12p70 is imperative for induction of a Th1 response,[71,72] and is enhanced by IL-27.[73,74] IL-1β, IL-6, and IL-23 are important for development of human Th17 cells,[82–85] that *per se* function to recruit neutrophils, but the role of Th17 cells during an Mtb-infection in humans has not yet been elucidated.[160] IL-10 acts as a general immunosuppressive cytokine, and plays a specific part during an Mtb-infection by inhibiting autophagy.[77,161] The remarkable functional plasticity of DCs to induce these different types of responses depends on which signals the DCs receive from the environment.[162] For example, single-triggering of toll-like receptor (TLR)2 and nucleotide oligomerization domain (NOD) 2 in moDCs gives rise to very low level IL-12p70, while the combined triggering gives intermediate amounts of IL-12p70.[28] Single-triggering of TLR4 on the other hand, gives higher amounts of IL-12p70,[97] while triggering of C-type lectin receptors (CLRs) dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209) and the mannose receptor (MR, CD206) inhibits TLR4-mediated IL-12p70 production, perhaps via increased IL-10 production.[22,26] It is well-established that Mtb contains ligands for both TLR2 and NOD2, by virtue of lipoproteins and muramyldipeptid (MDP), but it has also been suggested that Mtb contains non-canonical ligands for TLR4.[53,54,163] Furthermore, Mtb contains genomic DNA that can signal through intracellular DNA sensors TLR9 and stimulator of interferon genes (STING) to induce type I IFNs[50,164] that has been shown to boost IL-12p70 production in an autocrine manner.[165] It is therefore curious that Mtb only induces very small, almost undetectable amounts of IL-12p70 in moDCs.[28,55–57] Inhibitory

signaling by the Mtb-expressed CLR-ligands mannosylated lipoarabinomannan (ManLAM) and phosphatidylinositol mannoside 6 (PIM6) may partly explain the low IL-12p70,[22,26,166,167] but other factors, for instance relating to the host responsiveness are likely to play a part.

Studies on functional plasticity of moDCs have generally focused on cytokine-profiles and little is known about surface markers that can distinguish moDCs with different properties. Since the discovery of moDCs they have been described as being CD1a+ and CD14-low/negative.[107] However, interference during differentiation by IL-10, TLR ligands, and Mtb has been shown to generate DCs that do not express CD1a.[168–171] These CD1a-negative DCs were shown to be poor IL-12p70 producers, hence suggesting a linkage between CD1a-expression and IL-12p70 production.
In this report we analyzed how the expression-level of CD1a and other surface markers on moDC preparations affects the response to Mtb. We report that three different moDC subsets can be identified after Mtb-stimulation, one of them being specifically induced by Mtb.

2.3 Materials and Methods

Reagents. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: Mycobacterium tuberculosis, strain H37Rv, γ-irradiated whole cells, NR-14819, Mtb, strain H37Rv purified peptidoglycan (PGN), NR-14853, and purified ManLAM from strain Mtb H37Rv, NR-14848. *E. coli* LPS (O26:B6) was purchased from Sigma Aldrich. Recombinant human IFN-γ (cat. # 285-IF), IL-17A (317-ILB), and IL-22 (782-IL) was purchased from R & D Systems. The following anti-human Abs were used in the studies: CD1a/PE (clone# HI149), HLA-DR/v500 (G46-6), CD86/v450 (2331(FUN-1)), PD-L1/PE-Cy7 (M1H1), DC-SIGN/FITC (DCN46), MR/APC (19.2), CCR2/AF647 (48607), CCR5/APC-Cy7 (2D7/CCR5) (all from BD Biosciences), CCR7/APC-eFlour780 (3D12), CD14/PE-Cy7 (61D3), CD45RA/APC (HI100) (all from eBioscience), CD16/FITC (3G8), CD40/PerCP-Cy5.5 (5C3) (both from Biolegend), and CD4/ECD (SFCI12T4D11) (Beckman Coulter).

Human blood samples. Human buffy coats were acquired from the blood bank at the National Hospital of Denmark (Rigshospitalet) collected from anonymous healthy donors. Blood samples were handled in accordance with guidelines put forward in the "Transfusion Medicine Standards" by the Danish Society for Clinical Immunology (www.dski.dk). The buffy coats were obtained from 450 mL whole blood donations in citrate phosphate dextrose anticoagulant. Whole blood was fractionated to separate the majority of erythrocytes and plasma. The buffy coat contains approximately 90% of the leukocytes from the 450 mL of blood, and was used within 5.5 hours upon blood draw.

Cell purification and culture. Peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats by Ficoll-Paque (GE Healthcare) density centrifugation. The PBMCs were labeled with magnetic-particle linked CD14 Abs and isolated using MACS (Miltenyi). Cells were cultured in RPMI 1640 (Lonza) containing 2% (volume to volume) L-glutamine (Lonza), 10% fetal bovine serum (FBS, Lonza), 1% penicillin, streptomycin (Lonza), 50 μM β-mercaptoethanol (Sigma-Aldrich) and 10 mM HEPES (Lonza). Cells were kept in a humidified 37°C, 5% CO_2 incubator. For differentiation of monocytes into immature DCs, the medium was supplied with 20 ng/mL (≥160 U/mL) recombinant human GM-CSF and 30 ng/mL (≥150 U/mL) recombinant human IL-4 (both CellGenix). The cells were grown in polystyrene 6 well plates (Nunc) at a concentration of $2*10^6$/mL. Monocyte cultures were supplied with fresh medium containing fresh GM-CSF and IL-4 on day 3, and non-adherent immature DCs were harvested on day 6. In some experiments CD1a-hi moDCs were enriched by MACS according to the manufacturer's instructions. The purity of CD1a-hi moDCs were >92% for all donors. Immature DCs were plated at a cell concentration of 10^6/mL and stimulated with 500 ng/mL LPS, 10 μg/mL PGN, 50 μg/mL ManLAM and 500 μg/mL Mtb (final concentrations) determined as optimal concentrations in dose-response experiments (data not shown). Right before adding Mtb, clumps of bacteria was disrupted through syringe shearing by passing the solution 10 times through continuously smaller needles in the order 18-, 21-, 23-, 25- and 27-gauge. Recombinant human IFN-γ, IL-17A, and IL-22 was added at 100 ng/mL.

ELISA. ELISA was performed according to the manufacturer's instructions. Supernatants of stimulated and unstimulated DCs were harvested after 24 hours and kept at -

40°C until analysis. The following ELISA kits were used in this study: human IL-1β, IL-6, IL-10, IL-12, IL-27, IFN-γ (all R&D Systems), and IL-23p19p40 (eBioscience).

Flow cytometry. Flow cytometry was performed according to standard procedures. The wash and staining buffer consisted of PBS with 1% FBS and 0.1% Na-azid (Sigma-Aldrich). Before staining, the cells were incubated for 10 minutes in staining buffer with 2% allogeneic human AB plasma (Rigshospitalet) to limit non-specific binding of Abs. Monocytes were analyzed right after isolation, immature moDCs were analyzed right after harvesting, and stimulated/unstimulated moDCs were analyzed after 24 hours.

CD4+ naïve T cell co-culture. PBMCs were enriched for CD3+ cells by Dynabeads FlowComp human CD3-kit (Invitrogen), which enables removal of magnetic labeled Abs before cell sorting. Subsequently CD3-enriched PBMCs were labeled with antibodies against CD4/ECD and CD45RA/APC and sorted on MoFlo XDP (Beckman Coulter) according to CD4+CD45RA++. Purity of T cells were assayed on FACSCanto II, and were >97% for all donors. Prior to the co-culture moDCs were stimulated with the respective stimuli for 6 hours. moDCs were washed, counted, and added in a ratio of 1:20 to naïve T cells. T cells and moDCs were co-cultured for 6 days.

Statistics. All data was tested for a Gaussian distribution using a D'Agostino & Pearson omnibus test. Data that did not follow a Gaussian distribution were either log-transformed or analyzed by a non-parametric statistical method. When comparing two samples, the student's paired t-test or the Wilcoxon matched-pairs signed rank test was used. When comparing more than two samples a repeated measures ANOVA, or the Kruskal-Wallis test with a Dunn's post-hoc test was used. All correlation analyses are based on Pearson or Spearman correlations. Prism 5 (Graphpad Software) was used to conduct the analyses.

2.4 Results

CD1a-high and CD1a-low moDCs respond differently to Mtb

To gain insight into the relationship between CD1a-expression and moDC response patterns towards Mtb, we generated moDCs from a range of donors and assayed if the level of CD1a differed between donors (Fig. 1A-C). Interestingly, we found that even though all moDCs expressed CD1a, a clear division of two moDC subsets based on CD1a could be made (Fig. 1A). One subset expressed CD1a on average at a 25-fold higher level than the other subset, while this subset expressed CD1a on average 53-fold greater than monocytes originating from the same donor (Fig. 1B). The two moDC subsets were labeled CD1a-high (CD1a-hi) and CD1a-low (CD1a-lo). Notably, different donors gave rise to very different levels of CD1a-hi (versus CD1a-lo) moDCs. The number of CD1a-hi moDCs followed a Gaussian distribution with a mean percentage of CD1a-hi at 63% (range 25-96%, n = 23). It has previously been reported that moDCs lacking the expression of CD1a are low IL-12p70 producers.[168–171] In line with this we correlated the cytokines produced after Mtb stimulation with the percentage of CD1a-hi DCs (Fig. 1C). We analyzed a range of DC cytokines traditionally used in directing the naïve Th response towards Mtb: IL-12p70 and IL-27 for Th1, IL-1β, IL-6, and IL-23 for Th17, and IL-10 as a general immunosuppressive cytokine. Correlation analysis showed that the strongest association between the percentages of CD1a-hi DCs and cytokine level was seen for the IL-12 family cytokines: IL-12p70, IL-23 and IL-27, where high level CD1a-hi increased the amount of cytokine produced (Fig. 1C). IL-1β was produced in slightly larger amounts and IL-6 in slightly smaller amounts when more CD1a-hi DCs were present. There was a small tendency towards the CD1a-lo subset inducing the most IL-10, although this was not statistically significant.

To further analyze the phenotype of the two CD1a-divided DC subsets, we measured a range of surface markers by flow cytometry, and gated on the CD1-hi and CD1a-lo to define their expression levels on the two populations. In line with what we found for cytokine expression levels, the CD1a-hi subset was found to express higher levels of immunogenic markers than the CD1a-lo subset upon Mtb challenge (Fig. 1D-F). The antigen-presenting and co-stimulatory molecules HLA-DR, CD86 and CD40 were upregulated on average 1.3, 2.9 and 2.9 respectively by the CD1a-hi subset

compared to the CD1a-lo subset. The lymph-node homing receptor CCR7, and the CLR DC-SIGN displayed similar expression levels in the two subsets. MR, programmed cell death 1 ligand 1 (PD-L1, CD274), CD16, CCR2, and CCR5 were all upregulated the most in the CD1a-lo DC subset, at a 1.9, 1.6, 3.0, 2.1, and 2.3 higher level respectively compared to the CD1a-hi DC subset.

To assay if the difference in phenotype between CD1a-lo and CD1a-hi moDCs could be conveyed in the level of IFN-γ-producing CD4+ naïve T cells, we conducted a co-culture experiment, using different levels of CD1a-hi moDCs from the same donors. After a 6-day co-culture, the IFN-γ produced by the naïve CD4+ T cells were shown to positively correlate with the number of CD1a-hi moDCs (Fig. 1G).

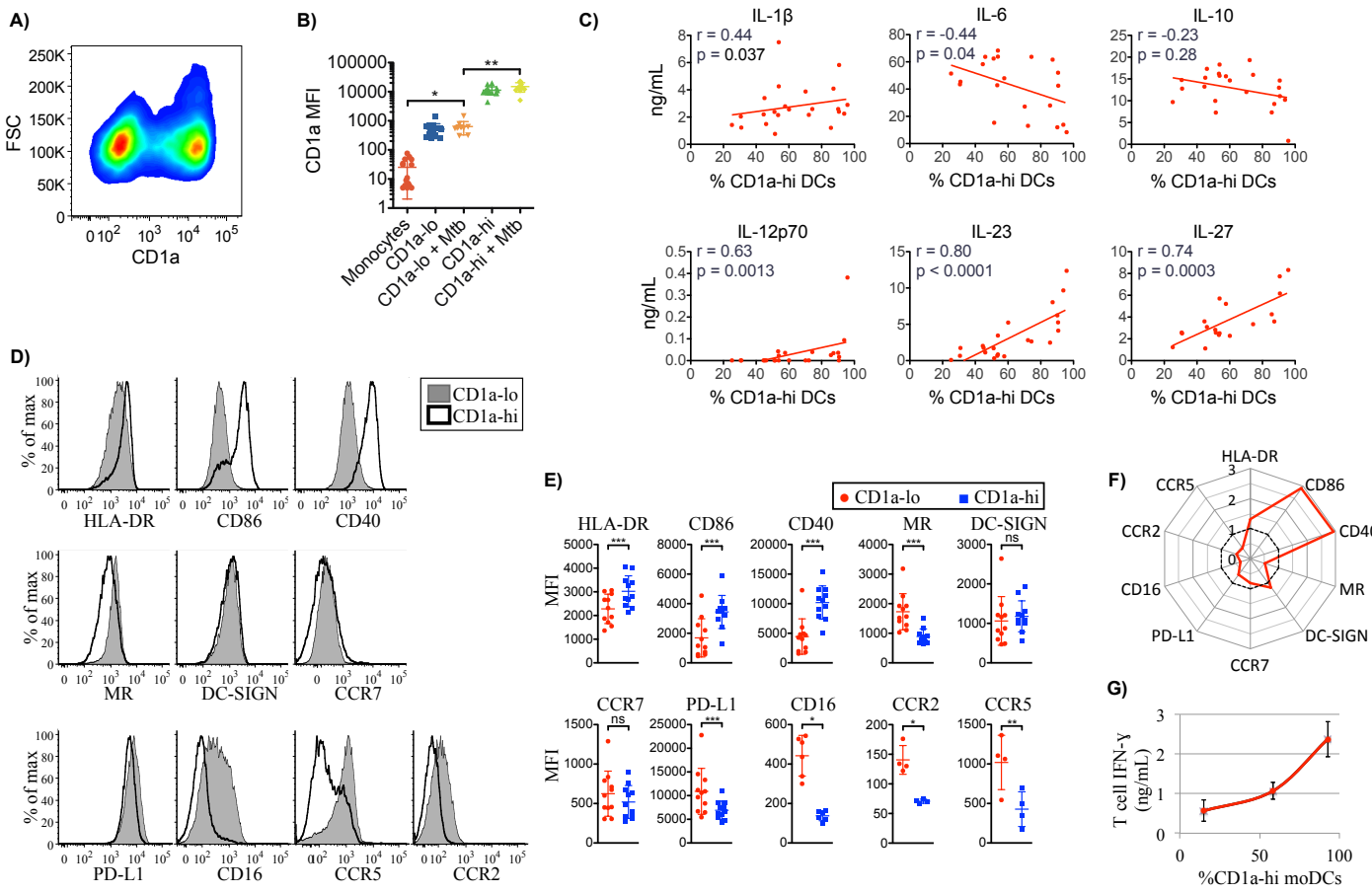

Figure 1: CD1a-dependent moDC response patterns against *M. tuberculosis*. Monocyte-derived DCs (moDCs) were stimulated with *M. tuberculosis* (Mtb) for 24 hours, and phenotypically analyzed by flow cytometry (A, B, D-F) or ELISA (C & F). A) FSC properties versus expression of CD1a on Mtb-activated moDCs (representative for n = 23). B) Monocytes and moDCs from the same donors were analyzed by flow cytometry to access CD1a expression levels. C) Supernatants from Mtb-

activated moDCs were assayed by ELISA and correlated with percentages of CD1a-hi DCs. Each dot represents one donor, and the line in the diagrams is the trend line shown for easier visual interpretation. D-E) Mtb-stimulated moDCs were gated into two populations determined by CD1a-expression. Ten other surface markers were assayed and mean fluorescence values (MFI) values extracted. Each dot in E) represents one donor, and the error bars represents SD. F) The ratio of CD1a-hi/CD1a-lo in expression level of the 10 surface markers was calculated for each donor, and the average of all donors (n = 4-11) plotted as shown. The numbers on the y-axis denominates the CD1a-hi/CD1a-lo MFI ratio, and the dotted line represents equal expression level on the two DC subsets. G) Different proportions of Mtb-primed CD1a-hi moDCs from the same donor were co-cultured with naïve CD4+ T cells for 6 days, and supernatant assayed for IFN-γ production. The data is representative for 2 independent experiments, and error bars represent SD of duplicates. Data in B) was tested for statistical significance by the Kruskal-Wallis test and Dunn's post-hoc test (n = 11). Data in E) were tested by a student's two-tailed paired t-test, or a Wilcoxon matched-pairs signed rank test (n = 4-11). Correlation analysis (in C) was done by a Pearson or a Spearman correlation test (n = 20-23). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CD1a-hi moDCs are relatively poor IL-12p70 inducers in response to Mtb

In the assayed range of donors it was apparent that a high amount of CD1a-hi moDCs were needed to produce any IL-12p70 at all in response to Mtb. To further elaborate on this, we compared the Mtb-induced response pattern in moDCs to moDCs stimulated with a prototypic IL-12p70-inducing ligand LPS (Fig. 2). When comparing with Mtb, especially IL-12p70, but also IL-27 was induced to the highest levels by LPS in CD1a-hi, and also in CD1a-lo moDCs (Fig. 2A, B). IL-1β and IL-23 were induced at the highest level by Mtb, while IL-6 and IL-10 was induced at a similar level by the two different stimuli. The pattern of cytokines was similar in CD1a-hi and CD1a-lo moDCs, but the levels varied, as already exemplified in Fig. 1C. To examine if these results could be explained by differences in surface marker display, we compared the expression level of a range of surface markers between Mtb- and LPS-stimulated moDCs gated on CD1a-hi and CD1a-lo moDCs (Fig. 2C,D). Most of the surface markers were expressed at similar levels, but notably LPS upregulated CD40 the most. Particularly, there was a large increase in CD40 on the LPS challenged CD1a-lo moDCs. However, the largest difference between LPS and Mtb activated moDCs was for CD14-expression. Both CD1a-hi and CD1a-lo Mtb-stimulated moDCs were found to upregulate CD14 to a higher level than in LPS (on average 21.5 and 13.6 fold increase on CD1-hi and CD1a-lo, respectively). The level of CD14 expression in Mtb-activated moDCs was also higher than the level found in unstimulated moDCs. Com-

pared to unstimulated moDCs, Mtb-activated CD1a-hi and CD1a-lo moDCs had on average a 6.6 and 6.3 fold higher expression of CD14.

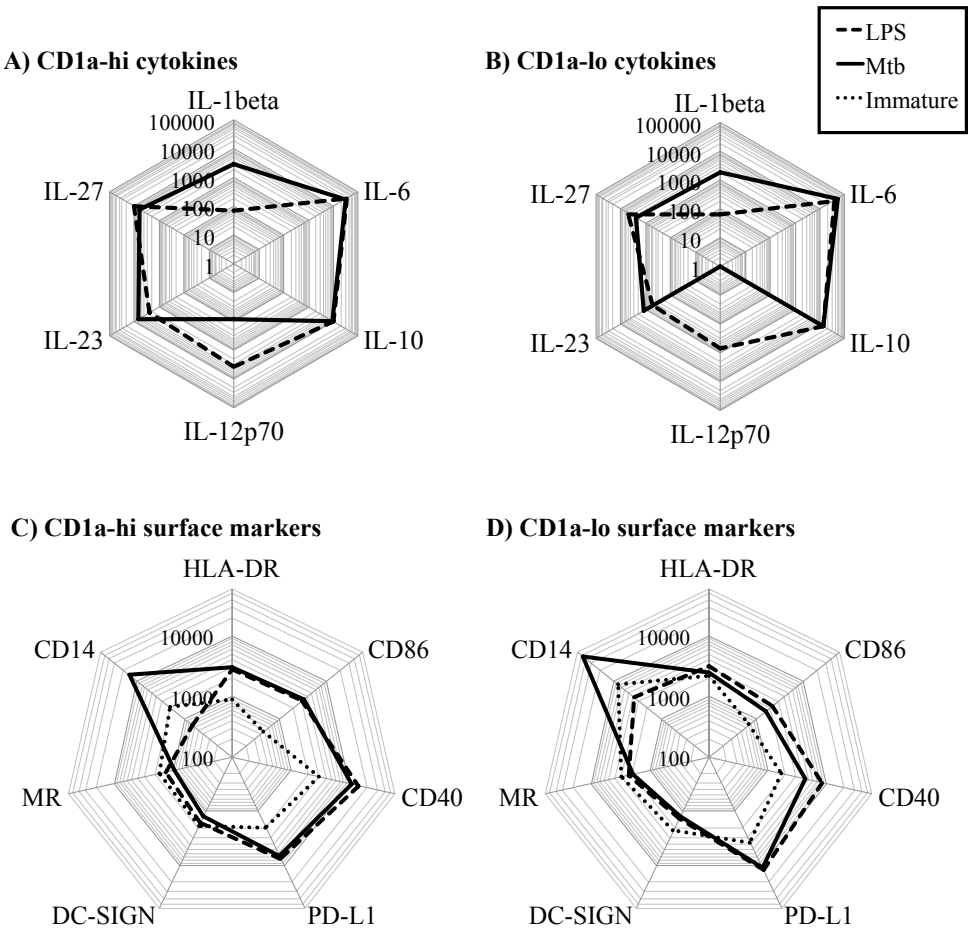

Figure 2: Response pattern-comparison of CD1a-hi and CD1a-lo moDCs between LPS and Mtb. moDCs were stimulated with Mtb and LPS, or left unstimulated for 24 hours and analyzed for surface marker expression by flow cytometry, and secreted cytokines by ELISA. For cytokine comparisons (A, B), the percentage of CD1a-hi DCs were noted and used to separate the DCs and thereby the response patterns into two groups based on <50% CD1a-hi expressing DCs (n = 7) or >80% CD1a-hi expressing (n = 6). For surface marker comparison (C, D) all donors were used (n = 13). The numbers on the log-axis in A and B represents pg/mL, while the numbers on the axis in C and D represents MFI.

Mtb propagates a CD1a-hi moDC subset with similar properties as CD1a-lo moDCs by upregulating CD14

To further examine the role of CD14-upregulation on Mtb-challenged moDCs, we used flow cytometry to assay the regulation (Fig. 3). Looking at immature moDCs, the two subsets based on CD1a-expression were clearly visible, but upon Mtb-exposure the two subsets shifted into three: CD1a(lo)CD14(hi), CD1a(hi)CD14(hi)

and CD1a(hi)CD14(lo) (Fig. 3A). In contrast, stimulation with LPS kept the two original DC populations, although CD14 was further downregulated. As IL-4 was previously reported to be responsible for downregulation of CD14,[172] and were left out of the medium during culture with LPS and Mtb (to avoid non-physiological bias), we also assayed the phenotype of moDCs left unstimulated for 24 hours to judge for CD14 shifting. In the unstimulated moDCs, three populations were apparent like for Mtb-challenged cells, but the CD14-expression was lower on unstimulated DCs compared to Mtb-stimulated DCs. Notably, the data illustrates that Mtb drives a polarization of moDCs that resembles that of unstimulated moDCs more than that of LPS, the latter exemplifying a prototypic Th1-promoting response type.

We also assayed the different subsets for CCR2 and CCR5 expressions to define the chemoattractive profile of cells upon Mtb exposure (Fig. 3B). Interestingly, the CD1a(hi)CD14(hi) moDC subset had a similar CCR5 profile as the CD1a(lo)CD14(hi) moDCs after Mtb exposure, while the CD1a(lo)CD14(hi) subset expressed more CCR2. CCR7-expression was not modified in any of the subsets by Mtb stimulation (data not shown). The CD14(intermediate)CD1a(lo) moDC-subset found after LPS exposure displayed a CCR-profile similar to the two CD14-hi subsets induced by Mtb, while the CD1a(hi)CD14(lo) subsets derived by Mtb or LPS activation also resembled each other besides from a tendency to a higher CCR5 expression on LPS activated moDCs (Fig. 3B). Another interesting finding for the Mtb-induced CD14-hi moDC subsets was an upregulation of CD16 expression, which was also apparent for LPS-activated moDCs (Fig 3B).

To examine if the CD14-hi moDC subset would affect cytokine production, we isolated CD1a-hi immature moDCs before stimulating with Mtb, measured the percentage of CD14(hi) moDCs induced by Mtb, and correlated this number to the levels of cytokines produced from Mtb-challenged CD1a-hi moDCs (Fig. 3C). Our data showed a clear inverse correlation between the number of CD14-hi moDCs induced by Mtb within the purified CD1a-hi population and the level of IL-12 family cytokines and IL-1β; generally, the higher the CD14-hi fraction, the lower production of IL-12 family cytokines and IL-1β. In line with this, the level of CD14-hi moDCs within the CD1a-hi moDC population also influenced the level of IFN-γ produced by CD4+ na-

ïve T cells co-cultured with moDCs (Fig. 3D). Production of IL-6 and IL-10 did, however, not seem to be affected by the Mtb-induced regulation of moDC subsets (Fig. 3C). Collectively, the data suggested a regulatory function of Mtb on moDCs resulting in increased CD14-hi expression, reduced IL-12 family cytokines, and diminished IFN-γ levels in CD4+ T-cells.

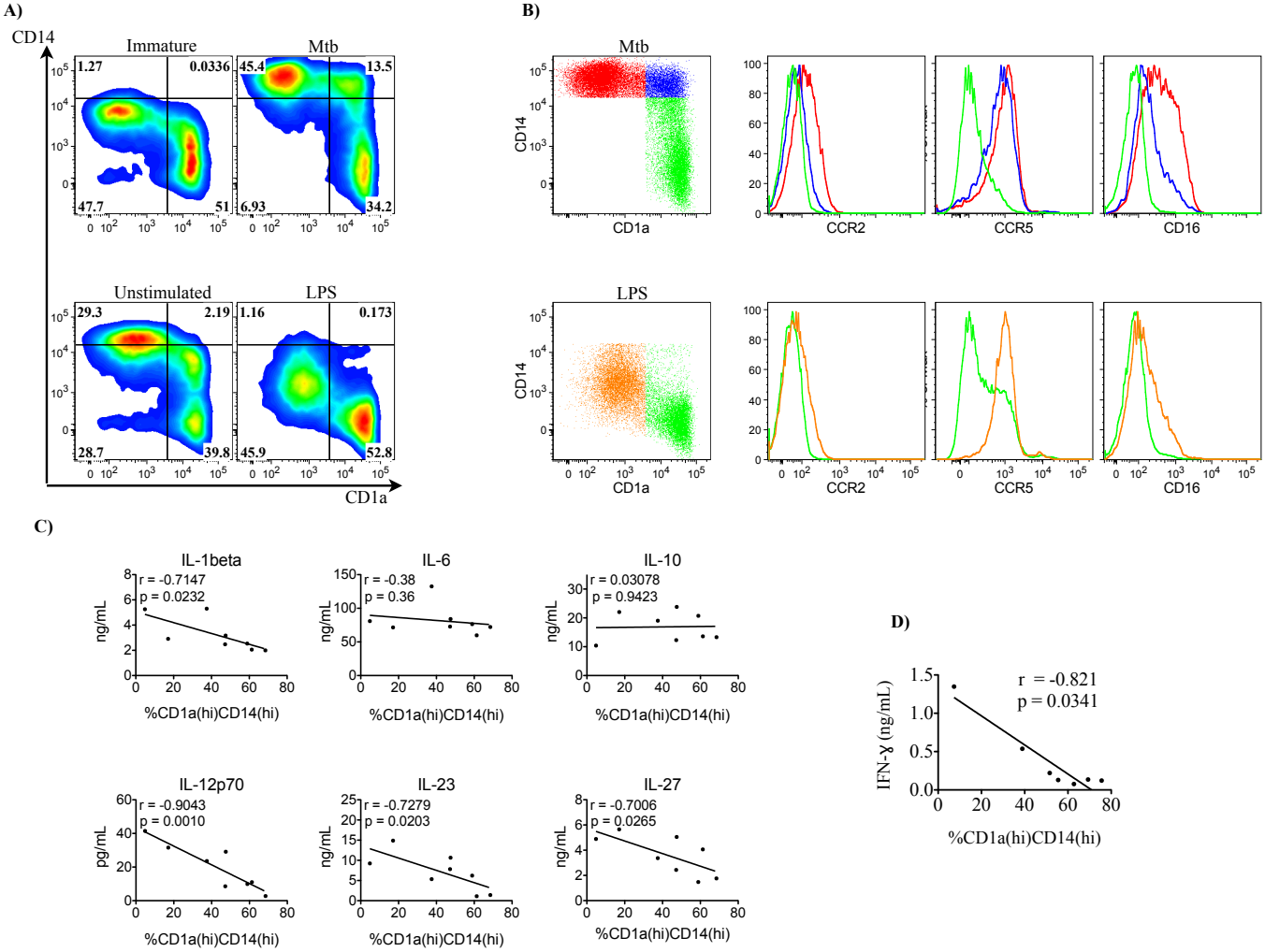

Figure 3: Induction of CD1a(hi)CD14(hi) moDCs by Mtb. A) Multicolor flow cytometry was used to assay difference in expression of CD14, between CD1a-lo and CD1a-hi immature moDCs, and moDCs stimulated with Mtb, LPS or left unstimulated. B) Flow cytometric characterization of Mtb- and LPS-stimulated moDCs. The color of the gate in the leftmost graph corresponds to the colors on the histograms to the right. C) CD1a-hi purified (>92%) were stimulated with Mtb, and assayed for CD14-expression by flow cytometry and cytokine production by ELISA. Correlation analysis between the number of CD1a(hi)CD14(hi) moDCs and cytokines produced are displayed. D) CD1a-hi purified moDCs containing various amounts of CD14-hi cells were co-cultured with T cells for 6 days. IFN-γ was assayed by ELISA, and correlated to %CD1a(hi)CD14(hi) moDCs. Correlation analysis was done by a Spearman correlation test (n = 7-8).

PGN and ManLAM partly promotes the CD1a(hi)CD14(hi) moDC subset

Mtb consists of a range of pathogen-associated molecular patterns (PAMPs), of which two of the most prominent are PGN and ManLAM. Therefore we sought to test if one or both of these PAMPs were responsible for the upregulation of CD14 in the CD1a-hi moDCs (Fig. 4). PGN-challenge of CD1a-hi moDCs resulted in distribution of the moDCs into two CD14-hi populations in a manner similar to Mtb, while ManLAM skewed the populations alike unstimulated moDCs (Fig. 4A). However, simultaneous stimulation with ManLAM and PGN induced a more profound increase in the overall CD14(hi) population in moDCs from most donors (Fig. 4A-B). ManLAM has previously been shown to increase IL-10 and decrease IL-12p70 production in LPS-triggered moDCs.[22,26] Therefore we speculated whether the regulatory increase in CD14-hi moDCs could explain these findings. To test this, we measured the cytokines produced by CD1a-hi moDCs stimulated with PGN and the combination of PGN and ManLAM (Fig. 4C, E). At the same time, we used multi-color flow cytometry to test if ManLAM induced any noticeable differences in the display of surface markers on moDCs (Fig. 4D-E). The differences in cytokines and surface markers were ordered according to the largest effects (Fig. 4E). Of the 6 cytokines tested, only IL-12p70 was consistently downregulated by ManLAM, and in general we observed great variations in cytokine levels induced by individual donor CD1a-hi moDCs (Fig. 4C). IL-1β, IL-6, IL-10, IL-23, and IL-27 were diversely regulated with some donors showing an increased expression, and others showing a decreased. ManLAM single simulation did not induce any cytokine production above detection level (data not shown). On the level of surface display of proteins (Fig. 4D, E), the two CLRs MR and DC-SIGN were found to be slightly reduced by ManLAM. CD40, CCR7, CD86, PD-L1, and HLA-DR also showed to be downregulated by ManLAM in most donors. CCR5 was regulated both up and down in a donor-dependent fashion. CCR2, CD16, and CD14 were upregulated for most of the donors, with CD14 having the highest average upregulation of all assayed cytokines and markers. Thus, our data demonstrate that Mtb-derived PGN and ManLAM collectively modify moDCs to program the Mtb-promoted CD14-hi, IL-12p70-low phenotype.

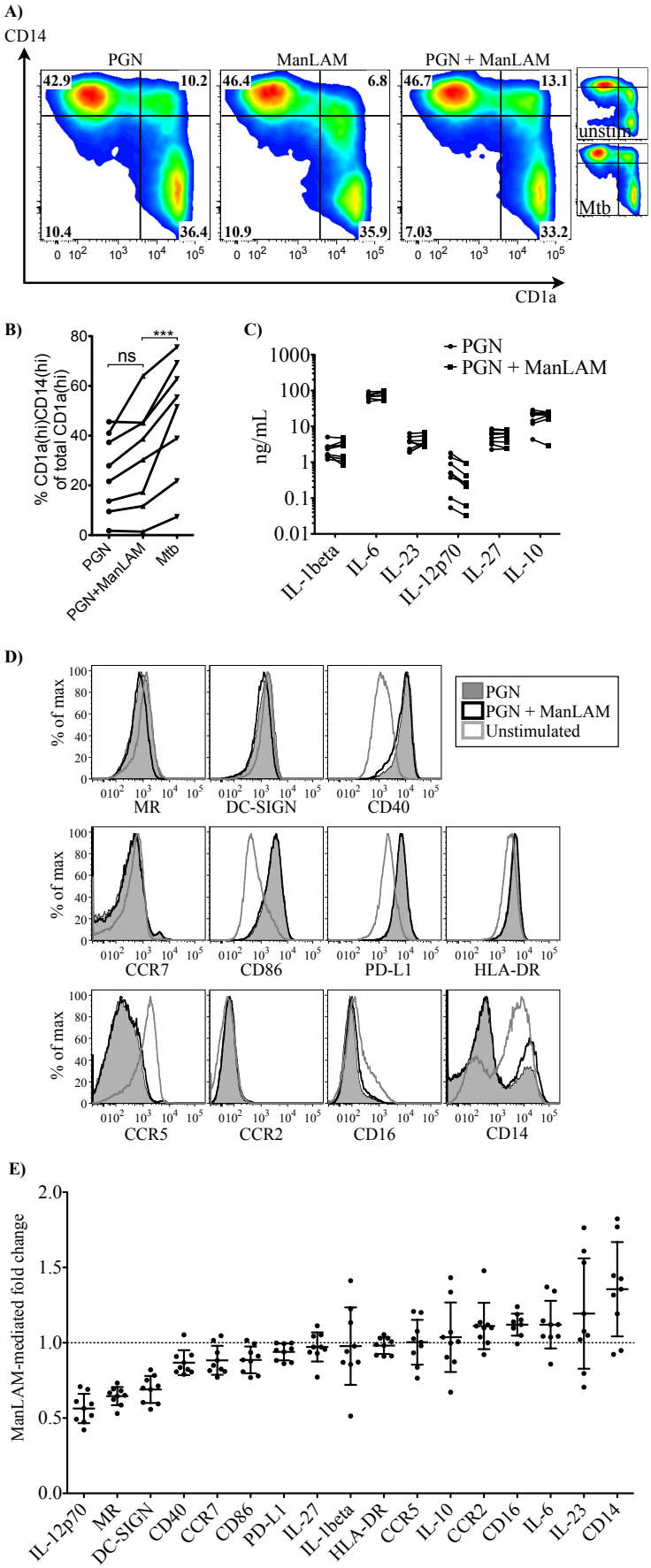
A)
CD14
PGN
ManLAM
PGN + ManLAM
42.9
10.2
10.4
36.4
46.4
6.8
10.9
35.9
46.7
13.1
7.03
33.2
unstim
Mtb
CD1a
B)
% CD1a(hi)CD14(hi) of total CD1a(hi)
ns
***
PGN
PGN+ManLAM
Mtb
C)
PGN
PGN + ManLAM
ng/mL
IL-1beta
IL-6
IL-23
IL-12p70
IL-27
IL-10
D)
% of max
PGN
PGN + ManLAM
Unstimulated
MR
DC-SIGN
CD40
CCR7
CD86
PD-L1
HLA-DR
CCR5
CCR2
CD16
CD14
E)
ManLAM-mediated fold change
IL-12p70
MR
DC-SIGN
CD40
CCR7
CD86
PD-L1
IL-27
IL-1beta
HLA-DR
CCR5
IL-10
CCR2
CD16
IL-6
IL-23
CD14

Figure 4: Role of PGN and ManLAM in CD1a(hi)CD14(hi) moDC induction. moDCs were stimulated with Mtb-derived PGN and ManLAM alone or in combination for 24 hours, and analyzed by flow cytometry (A, B, D & E) and ELISA (C & E). A) Representative flow cytometry plot for the division of moDCs according to CD1a- and CD14-expression patterns. The numbers in each gate represents the frequency inside the specified gate. B) Number of CD14-hi moDCs within the CD1a-hi gate for all donors (n = 8). C) Level of cytokines produced by PGN-single stimulation of CD1a-hi moDCs (left) or when combined with ManLAM (right). D) Representative flow histograms for ManLAM-mediated changes in PGN-induced phenotype of CD1a-hi moDCs. E) Collected ManLAM-mediated fold changes in PGN-induced phenotype for all assayed surface markers and cytokines ordered by increasing positive fold change. Each dot represents one donor, and the error bars represents standard deviation. Data in B was tested for statistical significance by a repeated measures ANOVA, ***$p<0.001$.

IFN-γ but neither IL-17A nor IL-22 inhibits induction of CD1a(hi)CD14(hi) in Mtb-challenged moDCs

γδT cells has been shown to produce the cytokines IFN-γ, IL-17A and IL-22 in response to Mtb-challenge.[173] To test if these three cytokines would modulate the induction of CD14-hi moDCs, we stimulated CD1a-hi DCs with Mtb alone or in combination with each of these cytokines (Fig. 5). IL-17A and IL-22 did not modulate the generation of the CD1a(hi)CD14(hi) moDCs induced by Mtb (Fig. 5A). IFN-γ on the other hand, almost completely inhibited the generation of Mtb-induced CD14-hi moDCs, resulting in a distribution of the moDCs into two subsets dispersed like those induced by LPS (Fig. 5A, B). IFN-γ added as a single stimulus also inhibited the incubation-induced generation of intermediate CD14 expression seen for unstimulated moDCs. IL-17A and IL-22 did not modify cytokine production from Mtb-activated moDCs, while IFN-γ modulated the production of most of the cytokines assayed (Fig. 5C & E). Most remarkably was the enormous increase in IL-12p70 production by IFN-γ addition, but IL-23 and IL-27 were also consistently increased in all donors. IL-6 was upregulated in most donors, while IL-1β was less constantly regulated amongst individual donors. IL-10 was also affected to a large extent, resulting in lower levels when IFN-γ was added. A range of surface markers was also assayed for modulation by IFN-γ (Fig. 5D-E). In addition to the extensive CD14-downregulation, CD16, CCR5, and CCR2 were also downregulated, but to a lesser extent. MR and CCR7 were not modulated by IFN-γ, while HLA-DR, PD-L1 DC-SIGN, CD40, and CD86 were increased. None of these markers were modulated by IL-17A or IL-22 (data not

shown), therefore suggesting that moDCs do not respond to IL-17A and IL-22, as opposed to IFN- γ.

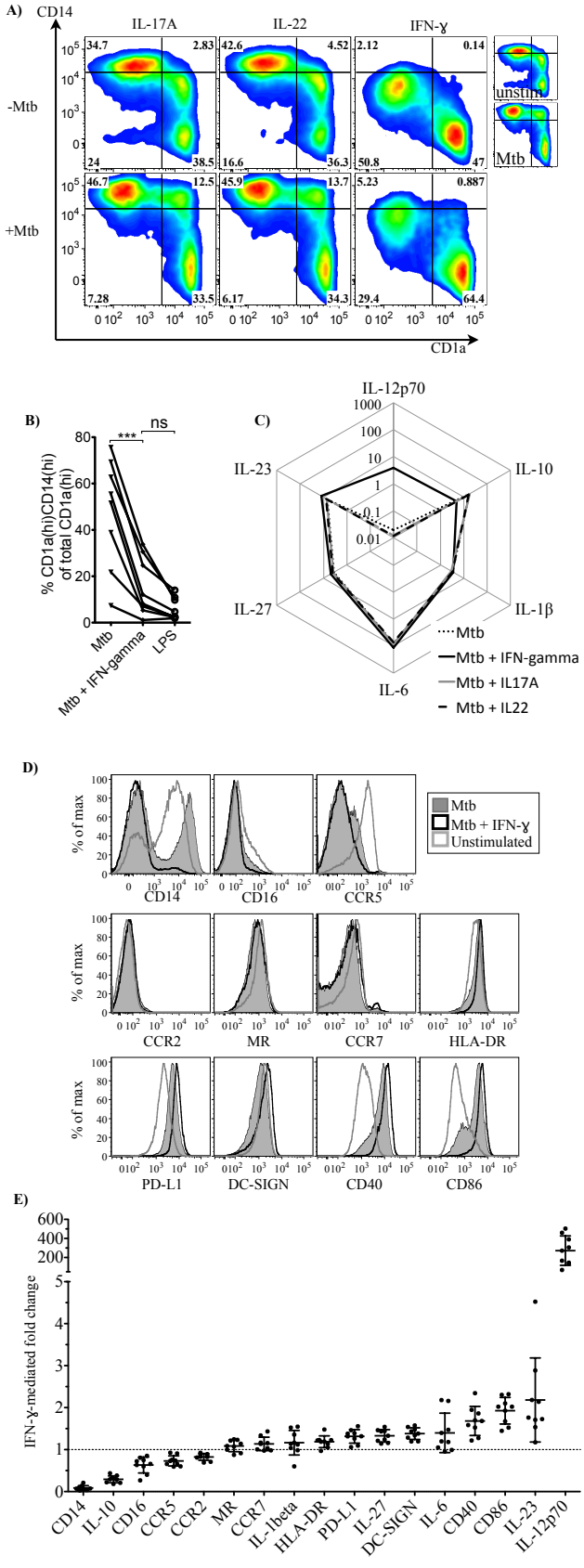

Figure 5: Role of IL-17A, IL-22, and IFN-γ on induction of CD1a(hi)CD14(hi) moDCs. moDCs were stimulated with Mtb and recombinant IL-17A, IL-22, and IFN-γ at 100 ng/mL alone or in the combination of Mtb + recombinant cytokine for 24 hours, and analyzed by flow cytometry (A, B, D & E) and ELISA (C & E). A) Representative flow cytometry plot for the division of moDCs according to CD1a- and CD14-expression patterns. The numbers in each gate represents the frequency inside the specified gate. B) Number of CD14-hi moDCs within the CD1a-hi gate for all donors (n = 8). C) Level of cytokines produced by CD1a-hi moDCs after Mtb-single stimulation or in combination with one of three recombinant cytokines. D) Representative flow histograms for IFN-γ-mediated changes in Mtb-induced phenotype of CD1a-hi moDCs. E) Collected IFN-γ-mediated fold changes in Mtb-induced phenotype for all assayed surface markers and cytokines ordered by increasing positive fold change. Each dot represents one donor, and the error bars represents standard deviation. Data in B was tested for statistical significance by a repeated measures ANOVA, ***$p<0.001$.

2.5 Discussion

Various subpopulations of DCs are known to co-exist in human tissues and may arise based on different progenitors, combined with a great plasticity within seeded DCs. Of current importance, both CD1a+ and CD1a- DCs are demonstrated to reside within human lungs.[111,114,174,175] Recent reports suggest that some of these DCs might derive from monocytes,[115,176] suggesting an *in vivo* relevance for moDCs in response to lung infections such as Mtb. Mtb single-stimulation of moDCs *in vitro* has been reported to induce close to no IL-12p70,[28,55–57] and it is believed that IFN-γ is needed for moDCs to produce IL-12p70 in response to Mtb.[28] The present data can help to explain the low level of IL-12p70 in Mtb-activated moDCs by demonstrating that mainly CD1a-hi moDCs induces IL-12p70 secretion. Furthermore, Mtb was shown to possess PAMPs that regulates the plasticity of these moDCs towards a phenotype with inverse display of high level CD14 expression and low level IL-12p70, thereby repressing propagation of IFN-γ-producing CD4+ T-cells. Intriguingly, this phenotypic shift in Mtb-challenged moDCs was observed to be fully inhibited by IFN-γ treatment, therefore suggesting that the role for IFN-γ in boosting IL-12p70 is partly to inhibit generation of this CD1a(hi)CD14(hi) moDC subset.

The combination of Mtb-derived PGN and ManLAM was partially responsible for this phenotypic shift in moDCs, and therefore it is possible that other microorganisms employing the same pattern recognition receptors could induce similar immune

modulations. Mtb PGN consists of MDP and γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP).[177] MDP is a ligand for the intracellular sensor NOD2,[178] while iE-DAP is a ligand for NOD1.[179] TLR2 also recognizes PGN by virtue of embedded lipoproteins such as LpqH, LprG, LprA, and PhoS1 for Mtb.[45] Most Gram-positive bacteria contains MDP and the TLR2-triggering ligand lipoteichoic acid (LTA), while NOD1-triggering is only promoted by selected Gram-positive bacteria.[179] In contrast, most Gram-negative bacteria trigger all three receptors, but concurrently trigger TLR4 by virtue of LPS, and thereby induce high IL-12p70 production.[180] Considering the phenotype of Mtb-activated moDCs it seems like the non-canonical TLR4 ligands present in Mtb[53,54] does not hold much stimulatory potential for moDCs, as a combined ligation of these with TLR2 and NOD1/2 would be expected to boost IL-12p70 similar to Gram-negative bacteria. *Bacillus subtilis* and *Listeria monocytogenes* are examples of two Gram-positive bacteria expressing ligands for all three receptors, while lacking TLR4-ligands. However, these two bacteria additionally express the TLR5-ligand flagellin, which induces copious amounts of IL-12p70 in moDCs.[97,181] It therefore seems likely that the specific combination of triggering mainly TLR2 and NOD1/2, in combination with certain CLRs, explains why Mtb is capable of repressing IL-12p70 production, and thereby evade immune clearance.

In contrast to PGN and Mtb, the prototypic IL-12p70-inducing stimuli LPS lead to further downregulation of CD14 compared to immature CD1a-hi moDCs. An explanation for this enhanced downregulation of CD14 by LPS could be due to receptor-internalization upon linkage. One observation, however, argues against this, as IFN-γ was also observed to mediate downregulation of surface CD14. A more likely mechanism is receptor-shedding induced by endogenous enzymatic activity, which has been demonstrated when monocytes were stimulated with LPS or IFN-γ.[182] It has been speculated that the physiologic role of CD14 shedding is to generate soluble CD14 needed for CD14- lymphocytes to respond to LPS.[183] It could also be speculated that the role of CD14-shedding or surface CD14 downregulation in the IL-12p70-producing moDC subset could be to limit an exaggerated immune response, in the sense that a cell already primed to produce IL-12p70 does not need to sense more LPS from the surroundings. An explanation for why CD1a-lo and CD1a(hi)CD14(hi) moDCs upregulate CD14 could be to sensitize these DCs to LPS-triggered apoptosis of terminally differentiated DCs, as described for murine DCs.[184] If this hypothesis

were true, it would also mean that the downregulation/shedding of CD14 would imply extended cell life to the CD1a(hi)CD14(lo) DC subset.

In addition to the currently identified linkage between surface CD14 and IL-12p70 production, the present data furthermore suggest that there is a linkage between CD40/CD86 expression and IL-12p70 production. Three points of evidence supports this notion: 1) CD1a-hi moDCs expresses IL-12p70, CD40, and CD86 at the highest level, 2) ManLAM inhibits CD40 and CD86 expression, as well as IL-12p70 production by PGN-activated CD1a-hi moDCs, and 3) IFN-γ boosts both CD40/CD86 expression levels, and IL-12p70 production by CD1a-hi moDCs. Since CD40 and CD86 are both co-stimulatory molecules during Ag-presentation, this could indicate that the highly-IL-12 family cytokine-producing moDCs are more prone to have proper interaction with naïve T cells, as demonstrated in a murine model system.[185]

CD1 molecules are structurally similar to MHC class I molecules, and is used to present lipids and glycolipids to T cells, and therefore play an important role in the immune response towards Mtb.[186] By inducing a subset of moDCs that has the ability to present Mtb-derived glycolipids (identified by CD1a-hi), but which at the same time lacks IL-12 family production and the ability to promote high level IFN-γ-production in CD4+ naïve T-cells (the CD14-hi subset), it appears that Mtb possess an ability to prime the immune system insufficiently. This phenomenon may be causative for the inadequate clearance of Mtb within lungs of infected individuals, but will require adequate *in situ* justification in future analysis. If moDCs has an *in vivo* relevance as reported by Varol *et al*. and Cheong *et al*.,[115,176] our data on CCR expression levels moreover suggest that only the CD1a(hi)CD14(lo) moDCs would migrate out of the lungs, by virtue of low CCR5 and CCR2 expression.[187–189] Likewise, it can be speculated that the CD1a-lo and CD1a(hi)CD14(hi) moDC subsets remains situated within the lungs, because of the lack of CCR5 downregulation. The role of these DCs could be to limit excessive immune response by virtue of the observed higher PD-L1 and CD16 expression levels. PD-L1 has been associated with tolerogenic DCs,[190] and signaling through CD16 has been shown to inhibit TLR4-mediated IL-12p70 production.[191]

In previous studies by others, it has not been emphasized that CD1a is differentially expressed on moDC preparations from different donors, but when looking through published data it is apparent that the CD1a-lo DC population is indeed present in some studies, as exemplified by the tail on the CD1a-histograms in these studies.[107,170,192,193] A few years ago a couple of research groups started reporting about CD1a- moDCs generated during standard differentiation.[194,195] These cells most likely correspond to the DCs we identify here as being CD1a-lo. For instance, like the present CD1a-lo moDCs, the CD1a- moDCs were characterized as having a higher expression of MR compared to the CD1a+ moDCs.[194] One of the most important findings for the CD1a- moDCs was their diminished ability to produce IL-12p70, which also corresponds with our findings. Besides from CD1a, moDCs also express CD1b, CD1c, and CD1d. These molecules were not included in our studies, because a previous report by Cernades *et al.*[195] demonstrating that these CD1-markers in contrast to CD1a were expressed at the same level on all moDCs (both CD1a- and CD1a+).

The different levels of CD1a-hi moDCs generated using the same culture conditions for a range of donors were quite curious, and we therefore examined the interference from several factors that could explain these differences. IL-10 has been reported to inhibit CD1a-expression on moDCs,[169] and recently it was suggested that within the CD14+CD16+ cell population a small blood DC population exists (named DC-10) that are natural producers of IL-10.[168] Therefore we analyzed the culture medium for an IL-10 contamination, but only found very small amounts, which furthermore did not correlate with the number of CD1a-hi DCs generated, or the amount of Mtb-induced IL-12 family cytokines (Fig. S1). Given that a possible IL-10 production by DC-10s could quickly be taken up by the cultured cells, and therefore not be found in the culture medium, we also FACS-sorted the monocytes in order to get completely rid of CD14+CD16+ monocytes and DC-10s. In contrast to the hypothesis that these cells might decrease the amount of CD1a-hi DCs, we found that after removal of CD16+ monocytes the number of CD1a-hi DCs was either at the same level or at a lower level than the DCs generated from the mixed population of monocytes (Fig. S2), thus neglecting this hypothesis. It has also been suggested that too little IL-4 or GM-CSF would affect differentiation into DCs,[196] so we tested the effect of increasing the concentration of these cytokines on the final CD1a-hi yield. In contrast to this prior notion, we did not observe any effect on the number of CD1a-hi DCs by increas-

ing the concentration or the ratio between IL-4 and GM-CSF (Fig. S3). The differentiation into CD1a-hi moDCs may of course be influenced by the recombinant cytokines used, and the specific medium composition, including the source of the FBS used. We always select every lab batch of FBS based on several factors including LPS-induced IL-12p70 levels, but other yet unknown exogenous factors may be more determinative for the resulting CD1a-display, if not the donor itself. Other groups have also reported differences in the level of CD1a-expression on moDCs generated from different donors. In a study by Gogolak *et al.*,[194] the same donors were tested twice to assay if *in vivo*-relevant regulatory pathways play a role in the various levels of CD1a. The authors only observed a ±5% change in CD1a+ moDCs when blood was drawn from the same donor at different times. This therefore suggests that the donor cell response in the *in vitro* culturing system is relatively stable, but greatly variable in-between donors, given that the range of CD1a-hi moDCs was 25-96% in our study and 0-100% in the study by Gogolak *et al*. The ability of Mtb to induce two different CD1a-hi subsets separated by CD14-expression, also varied extensively between donors (range 7.4-75.6%, n = 8) in the present study. It could therefore be suggested that the ability of monocytes and moDCs to differentiate into different subsets might be an intrinsically-encoded capacity within these cells that may be regulated at the gene level rather than by culture-dependent factors. The data presented here might help to explain why the capacity to develop protective immunity against mycobacteria is heterogeneously distributed among human beings,[197–199] but further *in vivo* studies are needed to make firm conclusions.

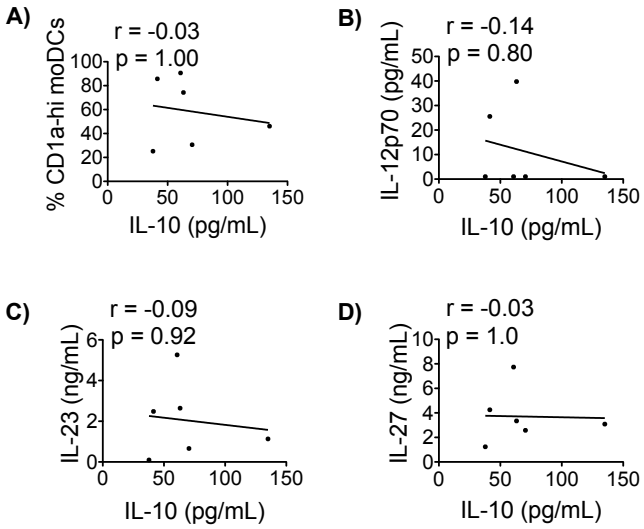

Figure S1: Effect of IL-10 present in culture medium on DC differentiation. moDCs were generated by the standard procedure. Prior to harvesting DCs at day 6, the supernatants of the culture medium were harvested and assayed for IL-10 by ELISA. The IL-10 contents were correlated to A) % CD1a-hi DCs assessed by flow cytometry and B-D) Mtb-induced IL-12 family cytokine production using Spearman's correlation test (n = 6). The line in the diagrams is the trend line shown for easier visual interpretation.

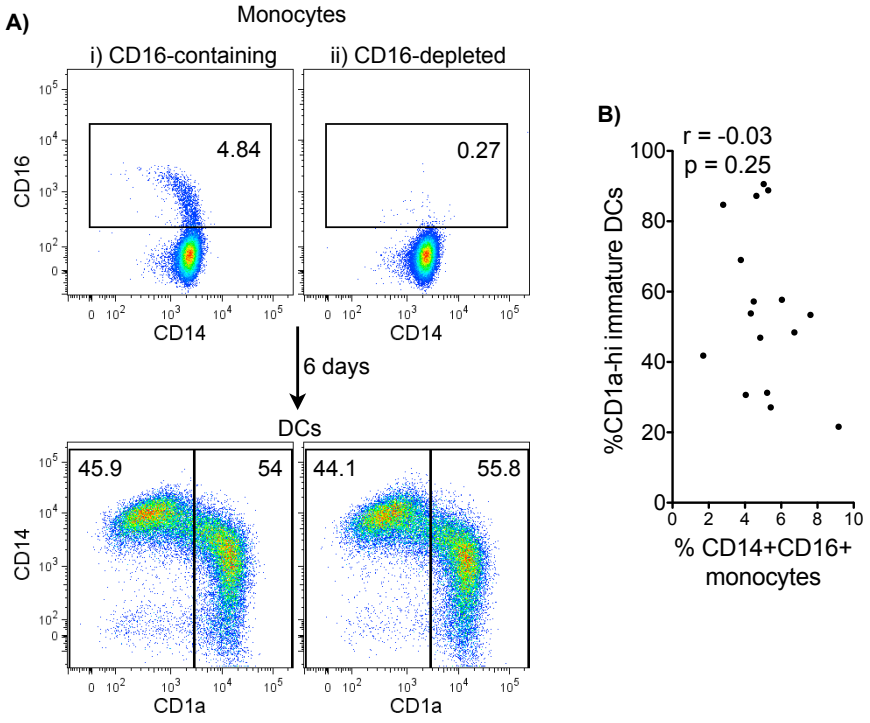

Figure S2: Effect of CD16+ monocytes on DC differentiation. A) Monocytes were enriched by CD14-positive selection followed by i) further purification of CD14+CD16+ monocytes, or ii) deple-

tion of CD14+CD16+ monocytes by cell sorting. The purity of the sorted populations was assayed by flow cytometry. Numbers in the FACS plots represents the percentage of cells inside the specified gates. After sorting, monocytes from i) and ii) were differentiated for 6 days, and assayed for CD1a expression by flow cytometry. The graph shows data from one representative experiment out of three. B) Correlation between the percentage of CD1a-hi DCs at day 6 and percentage of CD16+ monocytes in culture medium at day 0. Each dot represents one donor. Data was tested for a Gaussian distribution using the D'Agostino & Pearson omnibus test and correlation analysis was done by Pearson's correlation test (n = 16).

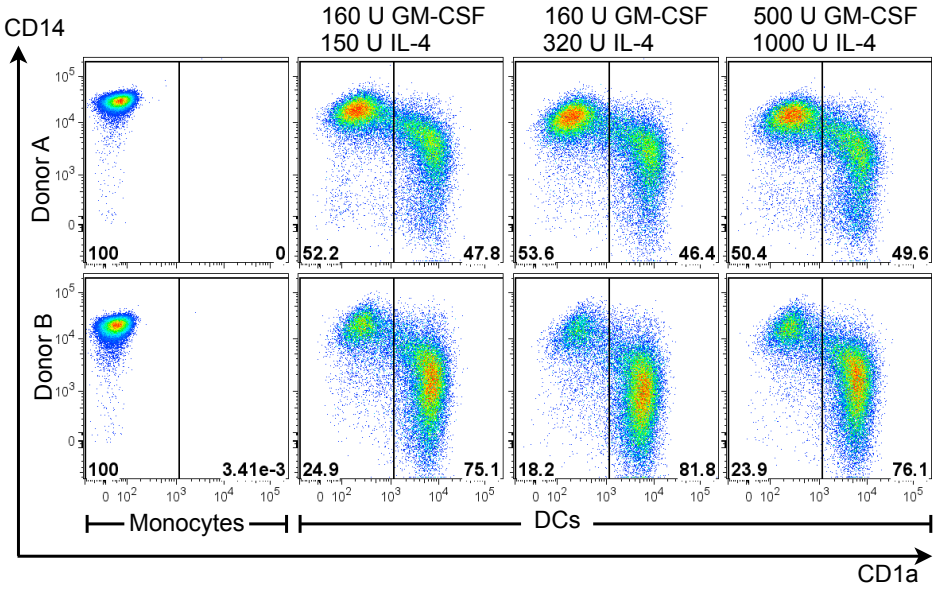

Figure S3: Effect of IL-4 and GM-CSF on DC differentiation. Monocytes were cultured in the indicated concentrations of IL-4 and GM-CSF for 6 days in order to differentiate into DCs. Expression of CD14 and CD1a on monocytes and moDCs from the same donors was assessed by flow cytometry. Numbers in the FACS plots represents the percentage of cells counted within the specified gates. Cells from donor A and B were isolated on the same day, and are representative of two similar experiments.

In summary, we have here demonstrated how Mtb exploits plasticity in moDCs to evade production of IL-12p70 needed for Th1-priming, and how the phenotype was fully recoded by exogenously administered IFN-γ.

3 Isolation of IL-12p70-competent human monocyte-derived dendritic cells

Authors

Jonas N. Søndergaard & Susanne Brix

3.1 Abstract

Diverse methodologies ranging from experimental immunological studies to immunotherapy involve the application of human monocyte-derived dendritic cells (moDCs). Considerable donor-dependent variations in the moDC production of IL-12p70 affect the outcome of these methodologies. It has been shown that moDCs generated under standard conditions develop into two subsets based on CD1a-expression with the CD1a+ moDCs being the main IL-12p70 producers. This has however not been generally accepted, which we show here is because the subset described as CD1a-negative does express CD1a, but at a lower level than the other subset. We further characterize the phenotype of these two subsets, showing that the CD1a-hi subset has a greater immunogenic phenotype, making this subset more suitable for immunotherapy. The two subsets have previously been separated by cell sorting, but as this technique is not available to many laboratories and has incompatibility with clinical settings, a more widely useable technique is warranted. Therefore we tested if magnetic-activated cell sorting is useful for the purpose, and show that it is possible to isolate IL-12p70-competent CD1a-hi moDCs to a <92% purity, irrespective of the starting purity.

3.2 Introduction

Monocytes have for many years been differentiated into dendritic cells (DCs) *in vitro* for the study of DC biology and utility in treatment of cancer.[200] One of the most studied DC-derived cytokines is IL-12p70 because of its importance in driving Th1-responses needed to amplify CD8+ T cell-mediated killing of tumor cells and intracellular infections.[72] Ever since the first report on the generation of immature DCs with good Ag-presentation capabilities using a combination of IL-4 and GM-CSF, monocyte-derived DCs (moDCs) has been described as CD1a-expressing.[107] Later it was shown that perturbed culture conditions induces the generation of CD1a-negative moDCs, which are not able to produce IL-12p70.[201] Certain research groups have reported that moDCs generated under standard conditions using FBS[194,195] or autologous human plasma[202] develops into both CD1a+ and CD1a- moDCs. Like the previously described CD1a- moDCs generated under perturbed culture conditions, the moDCs generated under standard conditions were largely incapable of producing IL-12p70. However, since most other research groups have reported that their moDC preparations expresses CD1a on all cells, this idea of a moDC subset lacking the ability to produce IL-12p70 generated under standard conditions has not been generally accepted. In this technical note we clarify that all moDCs generated under standard conditions express CD1a, but with a great variability in the levels of CD1a-hi and CD1a-lo cell subsets, influencing the capability of production of IL-12p70 and other cytokines important for Th-differentiation.

Previously CD1a- and CD1a+ moDCs have been isolated by fluorescence-activated cell sorting. The advantage of this is that it is possible to get a very high purity, but the downside is that it is time-consuming, and tends to be harsh on the DCs as compared to isolation using magnetic-activated cell sorting (MACS). Moreover, MACS is adaptable to clinical research settings. Therefore we tested whether it would be possible to use an existing CD1a-MACS kit intended for the isolation of Langerhans cells to purify the CD1a-hi moDCs from the CD1a-lo moDC population. The methodology is worthwhile for isolation of IL-12p70-competent DCs to be used for enhancing the effectiveness of cancer immune therapy as well as for a diversity of basic immunological research, e.g. cellular signaling studies were homogenous cell subsets are critical for understanding the molecular processes.

3.3 Materials and methods

Reagents. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: *Mycobacterium tuberculosis*, strain H37Rv purified peptidoglycan (PGN), NR-14853. *E. coli* LPS (O26:B6) was purchased from Sigma Aldrich. The following anti-human Abs were used in the studies: CD1a/PE (clone# HI149), HLA-DR/v500 (G46-6), CD86/v450 (2331(FUN-1)), PD-L1/PE-Cy7 (M1H1), DC-SIGN/FITC (DCN46), MR/APC (19.2) (all from BD), CD14/PE-Cy7 (61D3), (eBioscience), CD16/FITC (3G8), CD40/PerCP-Cy5.5 (5C3) (both from Biolegend).

Human blood samples. Human buffy coats were acquired from the blood bank at the Copenhagen University Hospital (Rigshospitalet) collected from anonymous healthy donors. Blood samples were handled in accordance with guidelines put forward in the "Transfusion Medicine Standards" by the Danish Society for Clinical Immunology (www.dski.dk). The buffy coats were obtained from 450 mL whole blood donations in citrate phosphate dextrose anticoagulant. The whole blood was fractionated to separate the majority of erythrocytes and plasma. The buffy coat contains approximately 90% of the leukocytes from the 450 mL of blood, and was used within 5.5 hours upon blood draw.

Cell purification and culture. PBMCs were isolated from human buffy coats by Ficoll-Paque (GE Healthcare) density centrifugation. The PBMCs were labeled with magnetic-particle linked CD14 Abs (Miltenyi) and isolated using MACS (Miltenyi). Cells were cultured in RPMI 1640 (Lonza) containing 2% (all percentages represent volume to volume) L-glutamine (Lonza), 10% fetal bovine serum (FBS, Lonza), 1% penicillin/streptomycin (Lonza), 50 μM β-ME (Sigma-Aldrich) and 10 mM HEPES (Lonza). Cells were kept in a humidified 37°C, 5% CO_2 incubator. For differentiation of monocytes into immature DCs, the medium was supplied with 20 ng/mL (≥160 U/mL) recombinant human GM-CSF and 30 ng/mL (≥150 U/mL) recombinant human IL-4 (both CellGenix). The cells were grown in polystyrene 6 well plates (Nunc) at a concentration of $2*10^6$/mL. Monocyte cultures were supplied with fresh medium containing fresh GM-CSF and IL-4 on day 3, and non-adherent immature moDCs

were harvested on day 6. Immature moDCs were plated at a cell concentration of 10^6/mL and stimulated with 500 ng/mL LPS and 10 µg/mL PGN (final concentrations) determined as optimal concentrations in dose-response experiments (data not shown).

Enrichment of CD1a-hi moDCs. Immature moDCs were carefully harvested on day 6 and washed 2X in PBSE-buffer consisting of PBS (Lonza), 2 mM EDTA (Sigma-Aldrich), and 2% FBS (Lonza). Cell pellet containing 25-50*10^6 immature moDCs were resuspended in residual PBS (~300 µL) and added 100 µL CD1a-Abs bound to magnetic beads (Miltenyi catalogue# 130-051-001), followed by a 30 min incubation at 4°C. Labeled cells were washed 1X in PBSE and resuspended in 3 mL degassed PBSE. MACS was performed by standard-procedure as described in the manufacturer's instructions, but in order to avoid column-blockage, degassed PBSE was used for all steps. The CD1a-lo fraction was collected as flow-through and the CD1a-hi fraction as column-bound. Before collection, the positive fraction the column was rinsed 3 times with 3 mL degassed PBSE.

ELISA. ELISA was performed according to the manufacturer's instructions. Supernatants of stimulated and unstimulated moDCs were harvested after 24 hrs and kept at -40°C until analysis. The following ELISA kits were used in this study: human IL-12 and IL-27 (both R&D Systems) and IL-23p19p40 (eBioscience).

Flow cytometry. Flow cytometry was performed according to standard procedures. The wash and staining buffer consisted of PBS with 1% FBS and 0.1% Na-azid (Sigma-Aldrich). Before staining, the cells were incubated for 10 minutes in staining buffer with 2% allogeneic human AB plasma (Rigshospitalet) to limit non-specific binding of Abs.

Statistics. All data was tested for a Gaussian distribution using a D'Agostino & Pearson omnibus test. Data that did not follow a Gaussian distribution were either log-transformed or analyzed by a non-parametric statistical method. Statistical significances were based on a student's paired t-test or a Wilcoxon matched-pairs signed rank test. Analysis was conducted using Prism 5 (Graphpad Software).

3.4 Results and discussion

The level of IL-12p70 produced by moDCs upon stimulation tends to vary considerably between donors. As expression of this cytokine is fundamental for various immunological settings such as DC immunotherapy and experimental studies of diverse microbial-induced phenotypes, we here demonstrate a method useful for isolation of pure IL-12p70-competent moDCs. Initially, we examined the reason for the contradiction in reports relating to the presence, or not, of CD1a-negative moDCs within *in vitro*-generated moDC preparations. By comparing the expression of CD1a on monocytes and moDCs from the same donors, we found that all moDCs expresses CD1a (Fig. 1a), but at two distinct levels, reflecting the two moDC populations that would correspond to the formerly reported CD1a- and CD1a+ subsets.[194,195] We analyzed both immature moDCs and LPS-activated moDCs (the latter is shown in fig. 1a), but activation did not alter the level of CD1a significantly. It could be argued that the CD1a-lo moDCs have a higher unspecific binding of the CD1a-Abs compared to the monocytes, as the LPS-activated moDCs are bigger. However, immature moDCs, which has a size closer to monocytes, also displayed the CD1a-lo moDC subset as having a much higher MFI than the monocytes they originated from (on average 53-fold increase in MFI, n = 11). Furthermore, the staining buffer was added 2% allogeneic human AB plasma, which should minimize unspecific binding significantly. Since all moDCs expresses CD1a, a more correct name would be CD1a-lo and CD1a-hi moDCs. Using this enumeration, we gated on the two populations for a range of independent donors, and found that the numbers of CD1a-hi moDCs were distributed very heterogeneously in the assayed population (Fig. 1b).

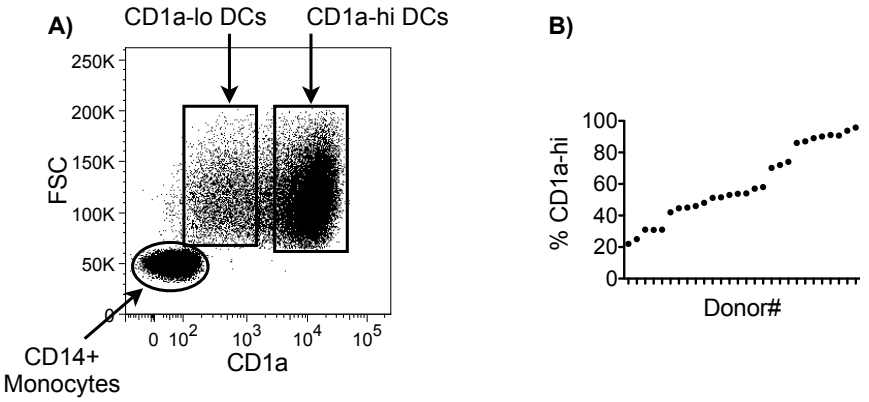

Figure 1: Diverse expression of CD1a on moDCs. A) Monocytes and moDCs from the same donor were analyzed for CD1a-expression by flow cytometry. The dot plot is representative for 11 donors. The expression levels of the moDCs and monocytes are overlayed within the plot (i.e. no monocytes are present within the moDC preparations). B) MoDCs were gated into CD1a-lo and CD1a-hi expressing subsets, and the % of CD1a-hi moDCs plotted for each donor (n = 28).

To further characterize the difference between the CD1a-lo and CD1a-hi moDCs, we gated on these two populations and compared a range of surface markers. At the immature state the CD1a-lo moDCs expressed the highest levels of HLA-DR, CD86, PD-L1, MR, DC-SIGN, CD14 and CD16 compared to the CD1a-hi moDCs (Fig. 2a). In immature CD1a-hi moDCs, only CD40 was expressed at the highest level. We also examined the LPS-activated phenotype of the moDCs (Fig. 2b). After stimulation, the CD1a-lo subset still expressed the highest level of PD-L1, MR, CD14 and CD16, while HLA-DR, CD86 and DC-SIGN, in contrast to the immature state, were expressed either at a lower level or at the same level as the CD1a-hi moDCs. CD40 was still expressed at the highest level on the CD1a-hi moDCs after stimulation. One noticeable observation for the immature DCs was the higher HLA-DR expression on the CD1a-lo subset. One explanation for this could be that during differentiation of monocytes into DCs some of the cells undergo apoptosis and is taken up by the DCs and therefore upregulate HLA-DR.[203,204] One could argue that the CD1a-lo moDCs are DCs that has taken up these apoptotic bodies, but one observation in our culture system clearly argues against this, as we usually obtained the lowest overall moDC number in cultures with the lowest percentage of CD1a-lo moDCs, and not the opposite. Therefore other factors than those pertaining to apoptotic regulation seem to be in play.

Fig. 2

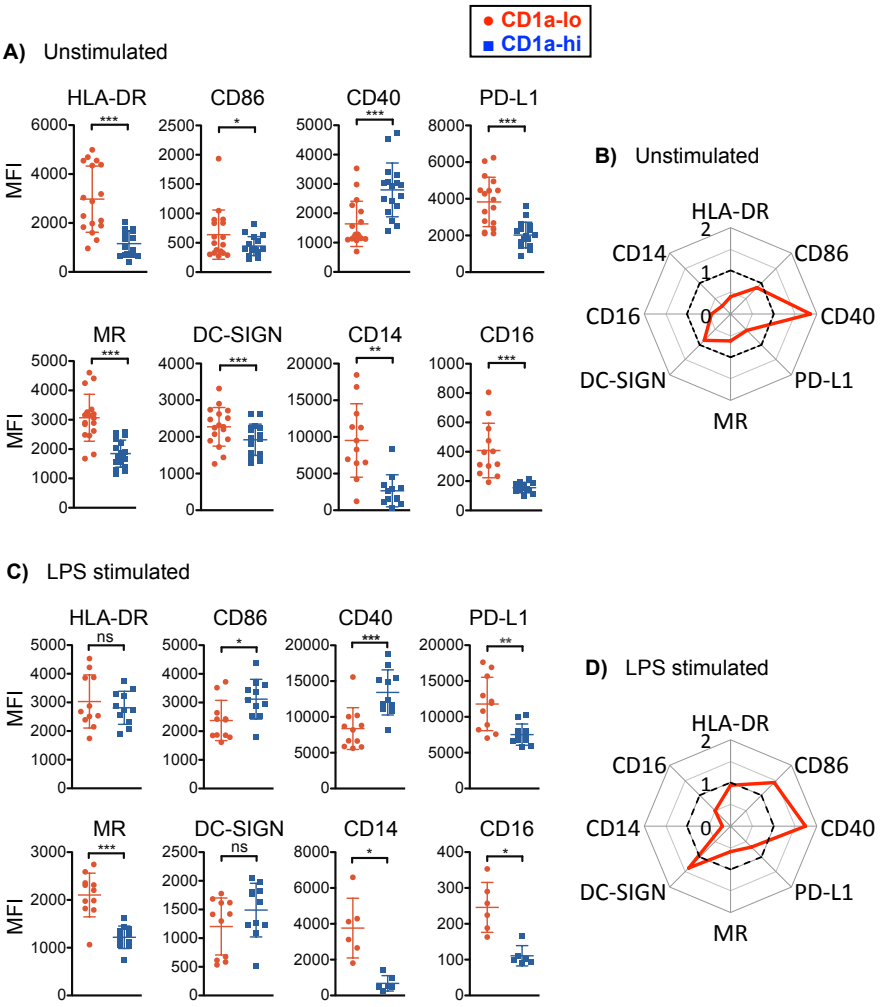

Figure 2: Characterization of differences in phenotype between CD1a-lo and CD1a-hi moDCs. Immature (A-B) and LPS-stimulated (C-D) moDCs were analyzed by flow cytometry and gated into two populations determined by CD1a-expression. Eight other surface markers were assayed and MFI values extracted. A and C: Each dot represents one donor, and the error bars represent standard deviation. B and D: The ratio of expression on CD1a-hi versus CD1a-lo moDCs of each surface marker was calculated for each donor, and the average of all donors is plotted. The numbers on the y-axis denominates the CD1a-hi/CD1a-lo MFI ratio, and the dotted line represents equal expression level on the two subsets. Data were tested for a Gaussian distribution using a D'Agostino & Pearson omnibus test. All surface markers except CD14 and CD16 for LPS-stimulated moDCs were analyzed for statistically significant differences by a student's two-tailed paired t-test (n = 11-17). CD14 and CD16 were tested for statistical significance by a Wilcoxon matched-pairs signed rank test (n = 6). *p<0.05, **p<0.01, ***p<0.001.

Interestingly, we found that at the immature state the CD1a-lo subset had a high expression of CD86 and HLA-DR without high level CD40, which possibly could lead to a regulatory state of interacting T cells.[185] Besides from having low IL-12p70 pro-

duction, tolerogenic DCs are reported to have a high expression of PD-L1,[190] which we also observed to be the case for the CD1a-lo moDCs. Another interesting observation for the CD1a-lo moDCs was the higher expression of CD16, as it has previously been reported that signaling through this receptor inhibits TLR4-mediated IL-12p70 production.[191] Based on these comparisons, it seemed apparent that the CD1a-lo moDC subset showed characteristics similar to those of tolerogenic DCs. Since both CD1a-lo and CD1a-hi subsets are present in all the moDC preparations generated for this study (n = 28), although to variable levels, it emerges that the protocols for experimental moDC studies should be selected cautiously. It must be kept in mind that the two moDC subsets differ in functionality and therefore may impact the outcome of the immunotherapy or the experimental study differentially. However, whether or not the general protocol for moDC generation is applicable will depend on the objective of the study. If the goal of a study is to examine surface markers and cytokine expression levels upon TLR-induced activation, the standard protocol for moDC generation may be used, as long as the percentage of CD1a-hi moDCs is recorded per donor. For studies of intracellular cell signaling it is of vital importance to have as homogenous a cell population as possible in order to minimize background signaling, and therefore a post-differentiation isolation of e.g. pure CD1a-hi moDCs would improve the results. The ratio of CD1a-hi/CD1a-lo moDCs derived from each donor will also have major implications for cancer immunotherapy, as one could speculate that the injection of CD1a-lo moDCs would dampen the immune response against tumor-derived Ag instead of strengthening it; this will be due to a low level of IL-12p70, and a high basal and matured expression level of PD-L1. One way to immediately improve cancer immunotherapy protocols would be to isolate the CD1a-hi moDC subset, and use this for treatment, instead of the current combination of CD1a-lo and CD1a-hi moDCs. Therefore we tested if it is possible to use MACS for isolation of the CD1a-lo and CD1a-hi moDC subsets. Figure 3A shows an example of cells from two donors isolated on the same day. It is clear that the purity of the CD1a-hi moDCs are at a satisfactory level above 92% (Fig. 3A and 3C, n = 10), while the purity of the CD1a-lo population is at an unacceptable level (e.g. 17% for donor Y). Besides from being the main IL-12p70-producers, the CD1a-hi moDCs also produced the other two IL-12 family cytokines IL-23 and IL-27 at a higher level than the CD1a-lo moDC subset (Fig. 3B), further verifying the enhanced immunogenicity of this moDC subset. On average the technique gave a yield of 67% CD1a-hi moDCs compared to the maximal

number available after 6 days of differentiation (Fig. 3D). Therefore it must be considered whether purity or yield has the highest priority in each experimental setup, before adapting this protocol.

Of notice is that we have used FBS in our culture system, which cannot be used for the generation of clinical-grade DCs, but exchanging FBS with autologous human plasma should make this procedure clinically adaptable. Given the ease, speed and greater sterility of the magnetic isolation procedure compared to cell sorting, this may be applicable right away for clinical testing of improved immunogenicity of tumor-Ag loaded CD1a-hi moDC preparations.

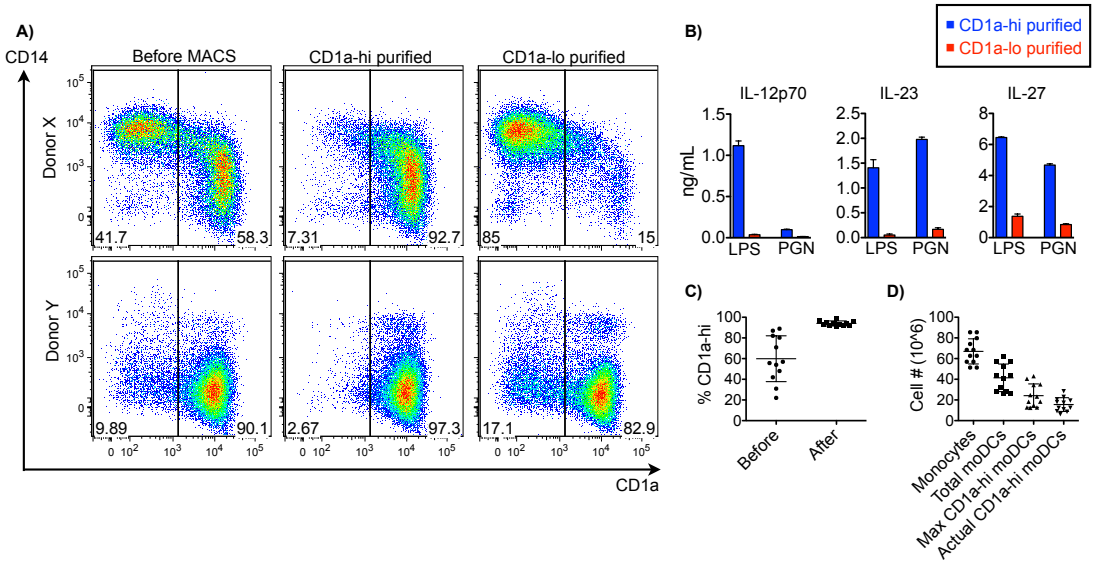

Figure 3: Isolation of IL-12p70-competent moDCs by MACS. After 6 days of differentiation, immature moDCs were harvested and labeled with CD1a-Abs linked to magnetic beads. The CD1a-hi moDC population was subsequently isolated by positive selection by MACS, while the CD1a-lo population was collected as flow-through. A) Purity of the different populations was assessed by flow cytometry. Numbers in the FACS plots represent the percentage of cells counted within the specified gates. The graphs show examples of isolation of CD1a-hi and CD1a-lo moDCs from two donors out of 12. B) Cytokine production measured by ELISA in the culture supernatants after 24 hours stimulation with LPS or peptidoglycan (PGN) of the two purified moDC populations. The plot is representative for the only 2 donors giving a satisfactory CD1a-lo purity (>85%). The error bars represent standard deviation of duplicates. C) Purity of the CD1a-hi moDC population before and after MACS. Each dot represents one donor (error bars = SD, n = 12). D) Yield of CD1a-hi purified moDCs. The plot also shows starting monocyte number, total moDC yield, and the maximum CD1a-hi yield calculated by multiplying the percentage of CD1a-hi moDCs before MACS with the total number of moDCs. Each dot represents one donor (error bars = SD, n = 12).

4 Kinome-wide analysis of human dendritic cells to deduce pathways involved in *M. tuberculosis*-mediated Th17-bias

Authors

Jonas N. Søndergaard, Erwin M. Schoof, Rune Linding & Susanne Brix

4.1 Preface

In the final manuscript about Mtb's interaction with moDCs the focus has been put on phosphorylation events induced in moDCs after Mtb exposure. This project is not yet finalized, as additional analysis of the kinome data, and verification in culture experiments is needed. The kinome data presented in this chapter has been generated based on a ratio between the level of kinase-activation of Mtb- and LPS-stimulated moDCs, in order to see if any Mtb-specific kinases could be identified. Further analysis will include 1) comparing stimulated phosphopeptide-enriched samples to total peptide sample, thereby enabling identification of additional Mtb/LPS-activated kinases, and 2) comparing all LPS-activated kinases to well-known signaling networks downstream of TLR4. Additional cell culture experiments will include verification of the role of the identified kinases in Mtb-DC interactions by using small molecule inhibitors, siRNA knockdown, and phosphoflow. Since this is the first time ever that anyone has used MS-based phosphoproteomics on human DCs, the finalized manuscript is intended for Nature letters. Therefore the manuscript has been prepared according to the guidelines of this journal, and hence has a different structure than the other papers included in this thesis.

4.2 Abstract

Dendritic cells (DCs) are at the frontline in the defense against the re-emerging pathogen *M. tuberculosis* (Mtb).[35–37] They detect the infection at the lung mucosal surfaces and subsequently initiate the adaptive immune response necessary for clearance of the infection.[17] At the very first contact, intracellular signaling cascades are induced, which will determine the phenotype of the DC, and ultimately shape how the immune response unfolds.[148] Only small fragments of the signaling cascades are known, and studies have so far been biased towards well-known pathways.[23,97–100,102–105] Here we

have for the first time used an unbiased kinome-wide approach to delineate the signaling induced by Mtb in human DCs. Twenty one Mtb-specific kinases were identified, which currently hold unknown roles in the immune response towards Mtb, and hence, need to be validated in cell culture experiments. The signaling induced by Mtb mediated an immune response directed at extracellular bacteria, as evidenced by a DC- and naïve CD4+ T-cell-phenotype biased towards Th17. Targeting the identified kinases might re-direct the immune response towards Th1-bias, thereby promoting clearance of latent Mtb. This could have therapeutic potential for the 2 billion people currently infected with this bacterium.

4.3 Results and discussion

The optimal immune response against an intracellular bacterium like Mtb includes an IL-12p70-driven Th1 response.[71,72] Purified CD1a-high, IL-12p70-competent monocyte-derived DCs were stimulated with Mtb and a prototypic IL-12p70-promoting bacterial stimulus (lipopolysaccharide, LPS). Interestingly, DCs stimulated with Mtb or LPS showed a very different cytokine response pattern (Fig. 1A). The prototypic Th1-inducing cytokine IL-12p70 was almost completely absent when DCs were stimulated with Mtb (on average 81-fold less compared to LPS). Likewise, the Th1-enhancing cytokine IL-27[73,74] was induced on average 2.2-fold less by Mtb. In contrast, the Th17-promoting cytokines IL-1β and IL-23[86] were induced on average 38-fold and 6-fold higher by Mtb compared to LPS. IL-6, which has been shown to enhance Th17 differentiation,[84] and the immunosuppressive cytokine IL-10[75,76] were both induced at similar levels by the two stimuli. Collectively, the DC cytokine response pattern suggests that Mtb promotes a Th17-bias, while LPS promotes a Th1-bias. To verify that DC phenotypes corresponded with the predicted T cell phenotypes, DCs were co-cultured with naïve CD4+CD45RA++ T cells isolated from allogeneic donors (Fig. 1B-C). Both extracellular and intracellular cytokine analysis of the DC-primed T cells showed that Mtb induces a stronger Th17-profile (7.5-fold extracellular, 1.7-fold intracellular) and a weaker Th1-profile than LPS (2.5-fold extracellular, 2.2-fold intracellular). Mtb furthermore induced a 5-fold higher IL-10 production from the naïve T cells compared to LPS.

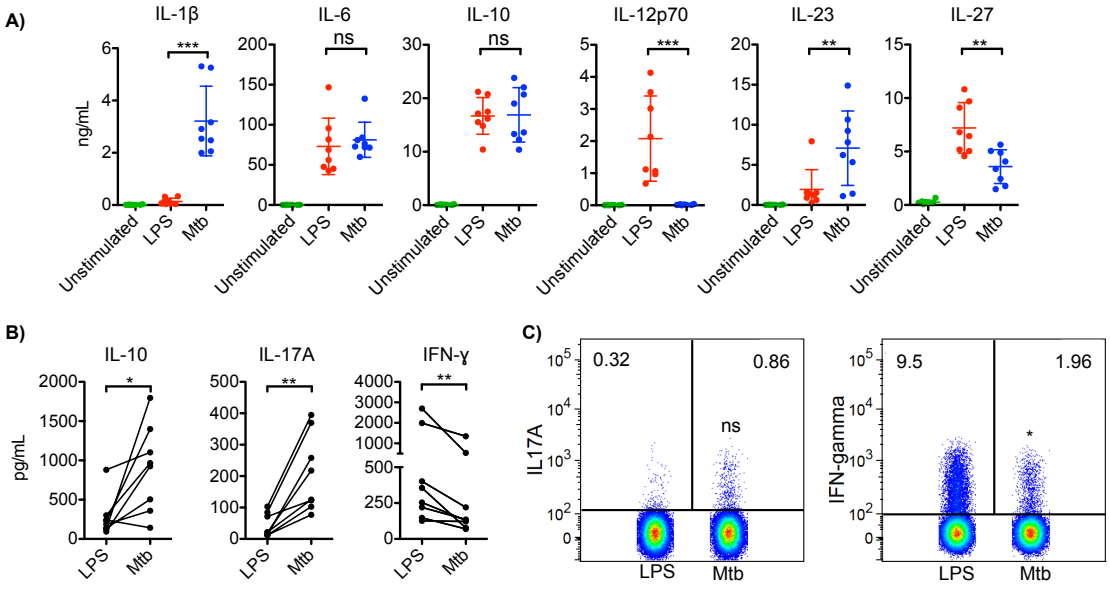

Figure 1: Mtb induces a DC phenotype promoting a Th17 bias. A) DCs were stimulated with LPS and Mtb or left unstimulated for 24 hours and supernatants were analyzed by ELISA. B-C) DCs were stimulated with LPS and Mtb for 6 hours, and subsequently co-cultured with allogeneic naïve CD4+CD45RA++ T cells for 6 days. Supernatants from the co-culture were harvested and analyzed by ELISA (B), and matured T cells were re-stimulated with PMA/ionomycin for 5 hours and analyzed by intracellular flow cytometry (C). The dot plot in (C) is representative of 8 donors, and statistics were conducted on all donors. Data were analyzed by a student's paired t test (n = 8).

At present, it is unclear if any surface markers on DCs particularly correlate with Th1 or Th17 responses. To assay this, we examined if there was any difference in the expression of a range of surface markers between LPS (Th1-promoting) and Mtb (Th17-promoting) (Fig. 2). Several of the assayed surface markers were regulated in the same manner by LPS and Mtb; HLA-DR, CD86, PD-L1 and CCR5. CD40 was more upregulated by LPS, which corresponds to a former report stating that CD40 upregulation is needed for IL-12p70 production.[185] The CLRs, MR and DC-SIGN were more downregulated by Mtb, which can be contributed to a ligation of these from Mtb-derived ManLAM and PIM6.[22,43,44] Interestingly, CD16, which has been shown to inhibit TLR4-mediated IL-12p70 production,[191] was upregulated the most by Mtb. CCR7 was curiously downregulated by Mtb, which could be due to the specific strain of Mtb (H37Rv) used for the studies,[205] or because of a general lack of CCR7 upregulation in monocyte-derived DCs.[206] A time course experiment showed that Mtb did not upregulate CCR7 to a higher level earlier than 24 hours (data not shown). Collectively, it could be suggested that CD40 and CCR7 are linked to Th1-responses, while

CD16, MR, and DC-SIGN are linked to Th17 responses. However, additional Th1 and Th17 promoting stimuli should be tested and compared to these results to make a firm conclusion.

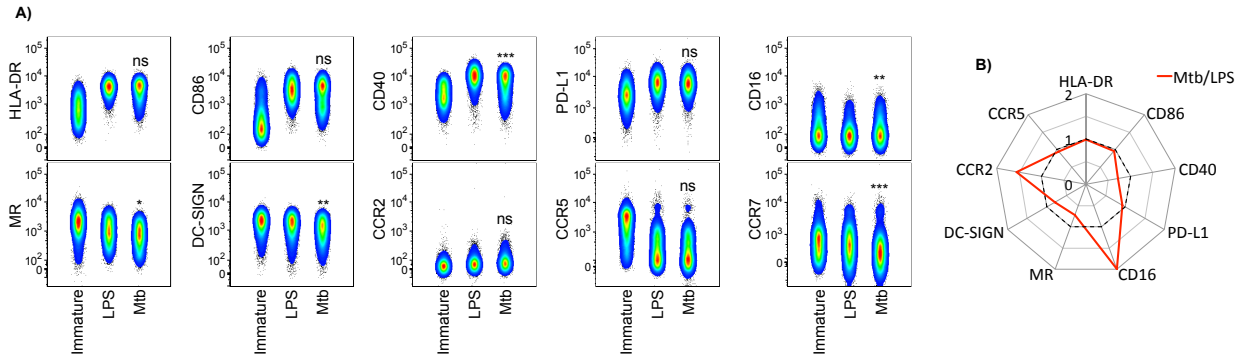

Figure 2: Differences in the DC surface marker phenotype between LPS- and Mtb-treatments. DCs were stimulated with LPS and Mtb or left unstimulated for 24 hours and analyzed by flow cytometry. A) Representative pseudo-color dot plots from one donor out of 8. MFI values were extracted and used for statistical analysis. The level of statistical significance, represented by asterisks over the Mtb stimulation, is based on comparison to the LPS-induced phenotype (n = 8). B) The ratio of expression on Mtb- versus LPS-stimulated DCs of each surface marker, averaged over the individual ratios. The numbers on the ordinate represents the Mtb/LPS MFI ratio, and the dotted line represents equal expression level induced by the two stimuli. Data was analyzed by a repeated measures ANOVA with a Tukey post-hoc test or a Friedman test with Dunn's post-hoc test.

Mtb contains pathogen-associated molecular patterns that previously have been demonstrated to induce IL-12p70,[28,53,54,163] thus arguing that Mtb actively inhibits IL-12p70 production in DCs. In order to assay if regulation of IL-12p70 is happening before or after gene transcription, the expression of *IL12A*, encoding IL-12p35, which is one of the two heterodimers constituting IL-12p70, was assayed in a time-course experiment (Fig. S1). The data clearly demonstrated that Mtb did not induce any up-regulation of *IL12A*, thus arguing that regulation of IL-12p70 is taking place pre-transcriptionally. In contrast to *IL12A*, Mtb did induce upregulation of *IL10*.

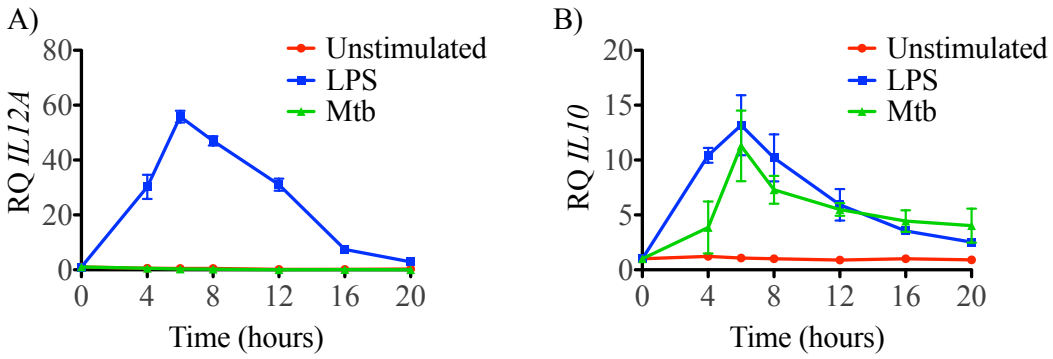

Figure S1: qRT-PCR time-course for upregulation of *IL12A* and *IL10*. DCs were stimulated with LPS and Mtb or left unstimulated for the indicated amount of time. RNA was purified, and relative quantity (RQ) of *IL12A* (A) and *IL10* (B) was determined by qRT-PCR on the basis of comparison to immature DCs harvested before initiating stimulations. Error bars represent SD for duplicates of separately stimulated DC preparations.

To obtain a better understanding of the processes of signaling involved in regulation of the IL-12p70 production in DCs by Mtb, a kinome-wide analysis was conducted. For comparison LPS was used because of its status as a prototypic IL-12p70-inducing stimuli. On average 356 phosphoepitopes were determined in the DCs after 30 minutes of stimulation (n = 3, Table S1).

Table S1: Numbers of regulated phosphoepitopes in all donors.

	Mtb-specific	Non-regulated	LPS-specific
Donor 1	47	268	12
Donor 2	49	282	32
Donor 3	55	286	38

In a preliminary assessment of the data, we focused on identifying kinases that were specifically activated by Mtb in comparison to LPS (Fig. 3, Fig. S2). A threshold of one SD from the mean ratio was used to define up- or down-regulation. In general Mtb activated much more kinases than LPS, which may be attributed to triggering of several pattern-recognition receptors (NOD1/2, TLR2, MR, DC-SIGN) instead of triggering of just TLR4. Three donors were assayed, and using the employed threshold, many of the assayed kinases did not overlap between donors (Table S2). The reason behind the small number of kinases activated consistently between donors can be explained by the different genetic backgrounds, possible meaning that signal-transduction is happening at different pace in individual donors. Different signaling cascades progressing from different pattern-recognition receptors triggered by Mtb

(NOD1/2, TLR2, MR, DC-SIGN) may also happen at different pace in individual donors. Therefore a time-study on a range of donors would be desirable to obtain the best understanding of the dynamics in signaling, and to enhance identification of which kinases are involved. Nevertheless, 21 kinases were identified to be specifically activated by Mtb in minimum 2 of the assayed donors, with 7 kinases being activated in all three donors (Table S2).

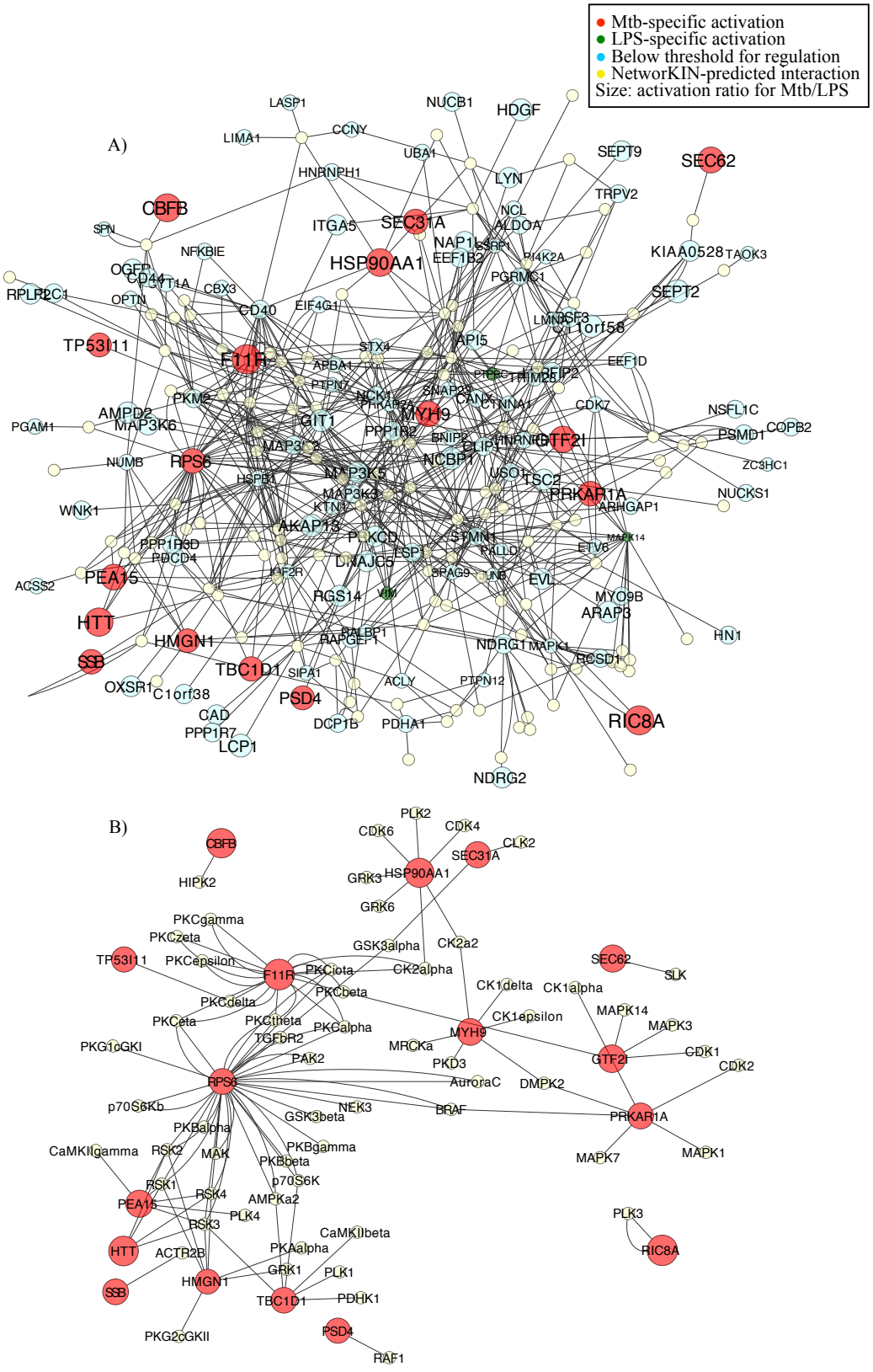

Figure 3: Identified and predicted kinases regulated by Mtb and LPS. DCs were stimulated with Mtb or LPS for 30 min. Phosphopeptides were enriched by TiO2 and assayed by LC-MS/MS. Identified peptides for the two stimuli were compared to each other to identify Mtb- and LPS-specific kinase activity. Interactions with other kinases were predicted using

NetworKIN. Identified kinases with no interactions are not displayed. A) Example of kinase network constructed from one donor. B) Isolated Mtb-activated kinases and directly-linked NetworKIN predictions.

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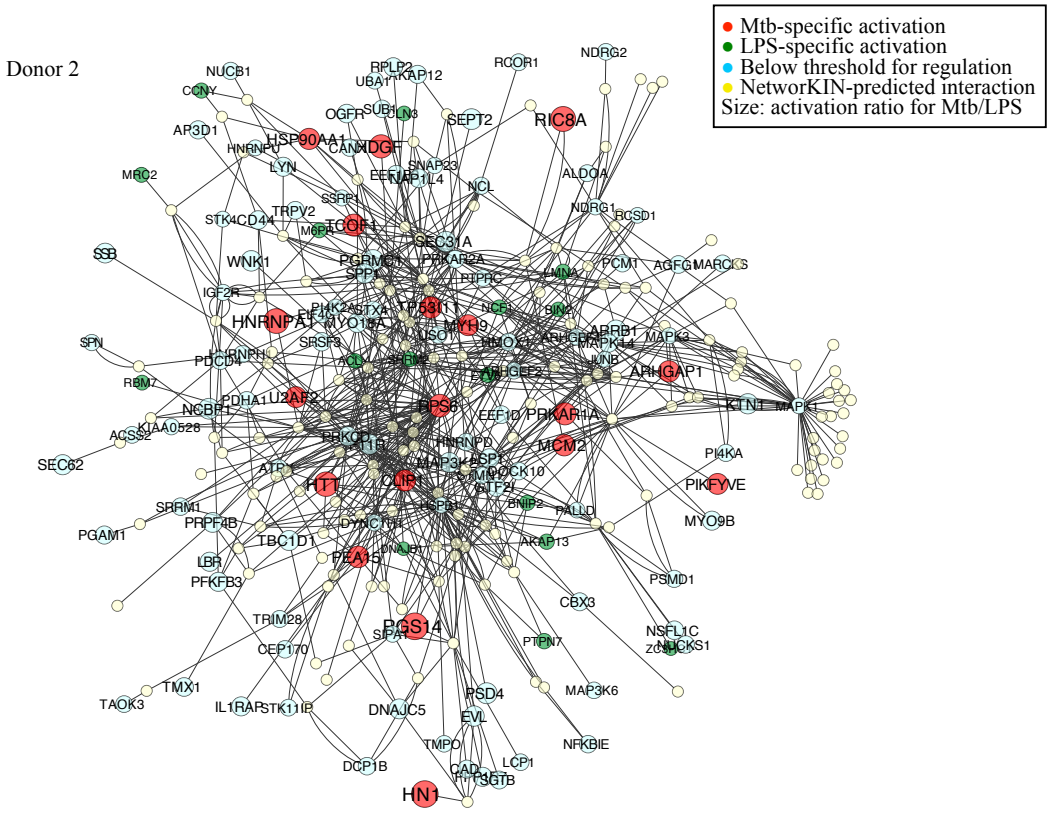

Figure S2: Identified and predicted kinases regulated by Mtb and LPS in additional donors. DCs were stimulated with Mtb or LPS for 30 min. Phosphopeptides were enriched by TiO2 and assayed by LC-MS/MS. Identified peptides for the two stimuli were compared to each other to identify Mtb- and LPS-specific kinase activity. Interactions with other kinases were predicted using NetworKIN. Identified kinases with no interactions are not displayed.

Table S2: Mtb-specific activated kinases identified in minimum 2 donors.

ID	Full name	Site	# of donors
MYH9	Myosin, heavy chain 9, non-muscle	S1943	3
HSP90AA1	Heat shock protein 90kDa α (cytosolic), class A member 1	S385	3
TP53I11	Tumor protein p53 inducible protein 11	S14	3
SPN	Sialophorin	T341	3
PEA15	Phosphoprotein enriched in astrocytes 15	S137	3
RPS6	Ribosomal protein S6	S235	3
EVL	Enah/Vasp-like	S333	3
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	S64	2
PSD4	Pleckstrin and Sec7 domain containing 4	S1019	2
SSB	Sjogren syndrome antigen B	S366	2
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	S627	2
U2AF2	U2 small nuclear RNA auxiliary factor 2	S79	2
GTF2I	General transcription factor IIi	S823	2
RIC8A	Resistance to inhibitors of cholinesterase 8 homolog A	S442	2
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	S6	2
HTT	Huntingtin	S1874	2
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, α	S83	2
HDGF	Hepatoma-derived growth factor	S181	2
KTN1	Kinectin 1	S75	2
SRRM1	Serine/arginine repetitive matrix 1	S695	2
TCOF1	Treacher Collins-Franceschetti syndrome 1	S1387	2

The kinases assayed to be specifically activated by Mtb have not previously been linked with TLR/NOD/CLR-signaling and could therefore be interesting new findings. The kinases might, however, also not play a role in IL-12p70 inhibition, and need to be validated before making any conclusions. The NetworKIN predicted interactions could give clues whether the data holds validity or not. There were 47 kinases predicted to have interactions with the 7 kinases consistently activated by Mtb in the 3 donors (Fig. 3B, Table S3). Among these was Raf-1, which previously has been shown activated upon DC-SIGN ligation,[101] and hence could participate in Mtb-induced signaling. In the study by Gringhuis *et al.*,[101] Raf-1 was shown to induce IL-

10, known to inhibit IL-12p70, and therefore the assayed kinases predicted to interact with Raf-1 might also play a role in IL-12p70 inhibition. Another predicted interaction was MAPK3, better known as ERK1. ERK1 has also been associated with signaling downstream of Mtb-activated pattern-recognition receptors, and shown to inhibit IL-12p70 production.[97,99,102,103]

Table S3: NetworKIN-predicted kinases directly interacting with Mtb-specific activated kinases identified in all donors.

ID	Full name
AMPKa2	5'-AMP-activated protein kinase catalytic subunit α-2
AuroraC	Aurora kinase C
CaMKIIβ	Ca2+/calmodulin-dependent protein kinase IIβ
CaMKIIγ	Ca2+/calmodulin-dependent protein kinase IIγ
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CK1α	Casein kinase 1α
CK1δ	Casein kinase 1δ
CK1epsilon	Casein kinase 1 epsilon
CK2a2	Casein kinase 2a2
CK2α	Casein kinase 2α
CLK2	CDC-like kinase 2
DMPK2	Dystrophia myotonica protein kinase 2
GRK3	G-protein-coupled receptor kinase 3
GRK6	G protein-coupled receptor kinase 6
GSK3α	Glycogen synthase kinase 3α
GSK3β	Glycogen synthase kinase 3β
HIPK2	Homeodomain interacting protein kinase 2
ICK	Intestinal cell (MAK-like) kinase
MAK	Male germ cell-associated kinase
MAPK3	Mitogen-activated protein kinase 3
MRCKa	Myotonic dystrophy kinase-related CDC42-binding kinase a
NEK3	NIMA (never in mitosis gene a)-related kinase 3
p70S6K	p70S6 kinase
p70S6Kb	p70S6 kinase b
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2
PDHK1	Pyruvate dehydrogenase kinase isozyme 1
PKBα	Protein kinase B α
PKBβ	Protein kinase B β
PKBγ	Protein kinase B γ
PKCα	Protein kinase C α
PKCδ	Protein kinase C δ
PKCeta	Protein kinase C eta

PKCiota	Protein kinase C iota
PKCtheta	Protein kinase C theta
PKD3	Protein kinase D3
PKG1/cGKI	cGMP-dependent protein kinase 1
PLK1	Polo-like kinase 1
PLK2	Polo-like kinase 2
PLK4	Polo-like kinase 4
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RSK1	Ribosomal S6 kinase 1
RSK2	Ribosomal S6 kinase 2
RSK3	Ribosomal S6 kinase 3
RSK4	Ribosomal S6 kinase 4

In summary we have here shown that upon interaction with human DCs, Mtb induces an immune response biased towards induction of Th17 with concurrent lack of Th1-induction, suggesting oppositely directed regulations. Certain Mtb-specific kinases were identified that could be involved in this regulation, but further cell culture testing is needed to validate the role of the individual kinases.

4.4 Method summary

CD1a-high monocyte-derived DCs were isolated as described by Søndergaard *et al.* (J. Immunol. Method., submitted 2012), and stimulated with Mtb or LPS for 30 min, 6 hours, and 24 hours for kinome-, T cell co-culture- and phenotype-analysis, respectively. DCs and T cells were subsequently co-cultured for 6 days. Phenotype of DCs and T cells were analyzed by flow cytometry and ELISA. For kinome analysis, DCs were lysed in a modified RIPA buffer. Proteins were precipitated, denatured, reduced and alkylated before being digested with LysC and trypsin. Peptides from unstimulated, LPS- and Mtb-stimulated DCs were differentially labelled on SepPack columns by dimethyl labelling,[207] and subsequently mixed together. Phosphopeptides were enriched by TiO2-based enrichment, and analyzed by LC-MS/MS on a Q Exactive (Thermo Scientific). Spectral matching was conducted using MaxQuant (version 1.2.2.5). Statistical analysis of MS data was done using R (version 2.15) and Python (version 2.6) and was based on normalized ratios of regulated peptides. All peptides with a regulation value larger than one SD from the mean were used for predicting kinase activity modulations using NetworKIN (version 2.0). Data was visualized by Cytoscape (version 2.8).

4.5 Methods

Reagents. The following reagents was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: Mtb, strain H37Rv, γ-irradiated whole cells, NR-14819. *E. coli* LPS (026:B6) was purchased from Sigma Aldrich. The following anti-human Abs were used in the studies: CD1a/PE (clone# HI149), HLA-DR/v500 (G46-6), CD86/v450 (2331(FUN-1)), PD-L1/PE-Cy7 (M1H1), MR/APC (19.2), CCR2/AF647 (48607), CCR5/APC-Cy7 (2D7/CCR5), DC-SIGN/FITC (DCN46), IL17A/PE (SCPL1362), IFN-γ/v500 (B27) (all from BD Biosciences), CCR7/APC-eFlour780 (3D12), (eBioscience), CD16/FITC (3G8), and CD40/PerCP-Cy5.5 (5C3) (both from Biolegend).

Human blood samples. Human buffy coats were acquired from the blood bank at the Copenhagen University Hospital (Rigshospitalet) collected from anonymous healthy donors. Blood samples were handled in accordance with guidelines put forward in the "Transfusion Medicine Standards" by the Danish Society for Clinical Immunology (www.dski.dk). The buffy coats were obtained from 450 mL whole blood donations in citrate phosphate dextrose anticoagulant. The whole blood was fractionated to separate the majority of erythrocytes and plasma. The buffy coat contains approximately 90% of the leukocytes from the 450 mL of blood, and was used within 5.5 hours upon blood draw.

Cell purification and culture. PBMCs were isolated from human buffy coats by Ficoll-Paque (GE Healthcare) density centrifugation. The PBMCs were labeled with magnetic-particle linked CD14 Abs and isolated using MACS (Miltenyi). Cells were cultured in RPMI 1640 (Lonza) containing 2% (volume to volume) L-glutamine (Lonza), 10% fetal bovine serum (FBS, Lonza), 1% penicillin, streptomycin (Lonza), 50 μM β-ME (Sigma-Aldrich) and 10 mM HEPES (Lonza). Cells were kept in a humidified 37°C, 5% CO_2 incubator. For differentiation of monocytes into immature DCs, the medium was supplied with 20 ng/mL (≥160 U/mL) recombinant human GM-CSF and 30 ng/mL (≥150 U/mL) recombinant human IL-4 (both CellGenix). The cells were grown in polystyrene 6 well plates (Nunc) at a concentration of $2*10^6$/mL. Monocyte cultures were supplied with fresh medium containing fresh GM-CSF and

IL-4 on day 3. Non-adherent immature DCs were harvested on day 6, and enriched for CD1a-hi expression by MACS as previously described (Søndergaard *et al.*, J. Immunol. Method., submitted 2012). CD1a-hi enriched DCs were plated at a cell concentration of 10^6/mL and stimulated with 500 ng/mL LPS and 500 µg/mL Mtb (final concentrations) determined as optimal concentrations in dose-response experiments (data not shown). Right before adding Mtb, clumps of bacteria was disrupted through syringe shearing by passing the solution 10 times through continuously smaller needles in the order 18-, 21-, 23-, 25- and 27-gauge.

CD4+ naïve T cell co-culture. PBMCs were enriched for CD3+ cells by Dynabeads FlowComp human CD3-kit (Invitrogen), which enables removal of magnetic labeled Abs before cell sorting. Subsequently CD3-enriched PBMCs were labeled with antibodies against CD4/ECD and CD45RA/APC and sorted on MoFlo XDP (Beckman Coulter) according to CD4+CD45RA++. Purity of T cells were assayed on FACSCanto II, and were >97% for all donors. Prior to the co-culture DCs were stimulated with the respective stimuli for 6 hours. DCs were washed, counted, and added in a ratio of 1:20 to naïve T cells. T cells and DCs were co-cultured for 6 days. Supernatants were harvested, and T cells were re-stimulated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) and 1 µg/mL ionomycin (Sigma Aldrich) for 6 hours. After 1 hour 10 µg/mL brefeldin A (BD Biosciences) was added.

ELISA. ELISA was performed according to the manufacturer's instructions. Supernatants of stimulated and unstimulated DCs were harvested after 24 hours and kept at -40°C until analysis. The following ELISA kits were used in this study: human IL-1β, IL-6, IL-10, IL-17, IL-12, IL-27, IFN-γ (all R&D Systems) and IL-23p19p40 (eBioscience).

Flow cytometry. Flow cytometry was performed according to standard procedures. The wash and staining buffer consisted of PBS with 1% FBS and 0.1% Na-azid (Sigma-Aldrich). Before staining, the cells were incubated for 10 minutes in staining buffer with 2% allogeneic human AB plasma (Rigshospitalet) to limit non-specific binding of Abs. Intracellular flow cytometry was conducted as previously described.[208]

Quantitative real time PCR. DCs were stored in RNAprotect Cell Reagent (Qiagen) at -80°C until analysis. RNA was extracted and purified from DC preparations using the RNEasy plus micro kit (Qiagen). cDNA was generated using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems). cDNA was quantified by quantitative real time PCR using TaqMan fast universal PCR master mix (Applied Biosystems) with the primers and probes from inventoried TaqMan gene expression assay mixes for *IL12A* (cat# Hs00168405_m1), *IL10* (Hs00174086_m1), *GAPDH* (Hs99999905_m1), and *ACTB* (β-actin, Hs99999903_m1). Amplification was analyzed on a 7900HT Fast RT-PCR system (Applied Biosystems). Relative quantity of *IL12A* was determined by using *GAPDH* as a housekeeping gene, and was similar to results obtained using *ACTB* (data not shown).

Phosphoproteomics. 10 million CD1a-hi human monocyte-derived DCs were rested for 60 minutes in a humidified 37°C, 5% CO_2 incubator before adding stimuli. After 30 minutes of stimulation, cells were quickly washed in 4°C PBS before being lysed in 2 mL of a modified RIPA buffer consisting of: 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% Na-deoxycholate, 1 mM EDTA, 5 mM β-glycerophosphate, 5 mM NaF, 1mM Na-orthovanadate (all from Sigma Aldrich) and Roche complete protease inhibitor cocktail. Proteins were precipitated in 80% acetone (Sigma Aldrich) at -20°C overnight. The protein pellet was dissolved in 2 mL denaturation buffer consisting of 6M urea, 2M thiourea and 10 mM HEPES (pH 8.0) (all from Sigma Aldrich), and protein concentration measured using the Bradford assay (Pierce). Concentration was around 1 mg protein/$5*10^6$ DCs. After normalizing the protein-concentration between samples, DTT (Sigma Aldrich) was added at 1 mM final concentration and incubated 60 minutes at room temperature. Subsequently proteins were alkylated using 5 mM (final) chloroacetamide (Sigma Aldrich) for 60 minutes at room temperature in the dark. Samples were next treated with 10 µg LysC (biochemical grade, Wako Bioproducts) for 4 hours at room temperature. Afterwards samples were diluted 4X in ultrapure H2O and adjusted to pH 8-8.5. 10 µg trypsin (modified sequencing grade, Promega) was added to each sample and incubated overnight at room temperature. Trypsination was stopped by adding trifluoroacetic acid (Sigma Aldrich) at a 2% final concentration, lowering the pH to about 2.5. The peptide mixture was centrifuged 5 min at 1800g to get rid of debris. Samples were dried down by vacuum centrifugation and reconstituted in 5% formic acid and differentially labeled by the

on-column dimethyl labeling method described in [207]. This step included purification of peptides on a SepPak column (Waters). After labeling, samples from each donor were mixed and phosphopeptides enriched using TiO2-based enrichment. 1-1.3 mg TiO2 beads (GL Sciences) were added and incubated for 30 minutes on lab rotor at room temperature. Beads bound to phosphopeptides were spun down and supernatant were enriched for phosphopeptides with a second round of TiO2 beads. This step was repeated giving a total of 3 phosphopeptide-enriched fractions. Peptides were separated from the TiO2-beads by a C8 StageTip (Thermo Scientific), and the peptide mixture was purified by a C18 StageTip (Thermo Scientific). LC-MS/MS was conducted on a Q Exactive (Thermo Scientific). Spectral matching was conducted using MaxQuant (version 1.2.2.5). Statistical analysis of MS data was done using R (version 2.15) and Python (version 2.6) and was based on normalized ratios of regulated peptides. All peptides with a regulation value larger than one SD from the mean were used for predicting kinase activity modulations using NetworKIN (version 2.0). Data was visualized by Cytoscape (version 2.8).

Statistics. All data was tested for a Gaussian distribution using a D'Agostino & Pearson omnibus test. Data that did not follow a Gaussian distribution were either log-transformed or analyzed by a non-parametric statistical method. Statistical significances were based on a student's paired t-test, a repeated measures ANOVA with a Tukey post-hoc test, or a Friedman test with a Dunns post-hoc test. Analysis was conducted using Prism 5 (Graphpad Software).

5 High-mannose glycosylation of HIV-1 gp120 and immune regulation in human plasmacytoid dendritic cells

Authors

Jonas N. Søndergaard, Lasse Vinner and Susanne Brix

5.1 Abstract

Background. Plasmacytoid dendritic cells (pDCs) play a vital role in activation of anti-HIV-1 immunity, and suppression of pDCs might mitigate early responses against HIV-1. HIV-1 gp120 high-mannose has been attributed immunosuppressive roles in human myeloid DCs. The present focus was to examine the role of HIV-1 gp120 high-mannose on pDC immunity.

Methods. Human peripheral blood pDCs were incubated with recombinant gp120s and an RNA analogue, or with inactivated or infectious HIV-1 strains. Intact HIV-1 was enzymatically treated to perturb gp120-mannose display. IFN-α production, CD40, CD86, HLA-DR, CCR7, PD-L1 expression levels and viability in pDCs were determined.

Results. Recombinant gp120s induced various phenotypes in pDCs, and the mannosylation degree on attenuated HIV-1 correlated inversely with IFN-α levels. However, perturbing the original mannosylation pattern on attenuated or viable HIV-1 strains failed to modulate viability, IFN-α, or surface marker expression in pDCs. In contrast, viable HIV-1 strains were found to confer differential, tropism-related effects on the pDC response, including profound cellular necrosis provoked by R5 strains.

Conclusion. Mannosylation of gp120 on viable HIV-1 did not contribute to immune regulation of pDCs. Selected, viable R5-tropic HIV-1, however, mediated non-mannose-dependent cellular atrophy of pDCs that might diminish innate immunity during early stage infection.

5.2 Introduction

Modulation of the human immune system by glycan-moieties from various pathogens is increasingly being recognized as a virulence factor. Particularly myeloid dendritic

cells (mDCs) have received much focus, and have been shown to be modulated by β-glucans, fucose, and high-mannose residues.[148] One of the most notorious pathogens of our time, HIV-1, is covered in a shell of glycan-moieties all linked to the membrane-associated glycoprotein gp120.[209–211] Recent data has indicated that a large part of these glycans consists of high-mannose,[212] which is capable of binding C-type lectin receptors (CLRs) present on DCs. Specifically, DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and the mannose receptor (MR) present on mDCs has been shown to interact with gp120 high-mannose.[213,214] Upon interaction, immunosuppressive responses are taking place, therefore suggesting that HIV-1 uses high-mannose-CLR ligation for evasion of a productive immune response in DCs.[148,149]

Plasmacytoid DCs (pDCs) are found in very scarce numbers in the human blood circulation (0.2-0.5% of peripheral blood mononuclear cells (PBMCs)), but play an utmost important role in initiating immune responses against HIV-1.[144,215] The main effector molecule produced by pDCs is IFN-α, which has been shown to limit HIV-1 replication in CD4+ T cells.[153] pDC-derived IFN-α furthermore activate mDCs,[140] and NK cells,[151] and polarize naïve CD4+ T cells towards Th1.[152] These three cell types are important for combating intracellular viral infections like HIV-1, underscoring the importance for pDCs during an HIV-1 infection. IFN-α production from pDCs is initiated by triggering of TLR7 or TLR9, with the former being responsible for recognizing HIV-1 by virtue of its' genomic ssRNA.[141] pDCs also express the CLRs BDCA-2 and DCIR,[19,21] but currently the glycan molecular patterns recognized by these receptors are unknown. DCIR has, however, been shown to bind HIV-1 most likely through gp120,[216] and recombinant gp120 has been shown to ligate to a cell line transfected with BDCA-2.[147] Activation of BDCA-2 and DCIR by Ab-crosslinking has been shown to inhibit TLR9-mediated IFN-α production in pDCs.[19,21] Similarly recombinant gp120 has been shown to inhibit TLR9-mediated production of IFN-α,[147] but curiously no gp120-mediated inhibition in TLR7-triggered pDCs was observed in this study. This latter, and all other studies conducted on the immune-suppressive effect of HIV-1 high-mannose have used recombinant gp120. Different response types are demonstrated dependent on which gp120 was employed.[149] An explanation for this was recently provided by Raska et al., reporting that the expression system used to produce the recombinant gp120 protein is greatly influencing the glycosyla-

tion pattern on gp120.[159] Therefore it is desirable to study the original mannosylation pattern on HIV-1 in order to make firm conclusions on the immune-regulatory abilities of viral-exposed gp120.

In this study we tested if human pDC immunity is modulated by high-mannose from a range of recombinant gp120s, AT-2 attenuated HIV-1 strains, and viable HIV-1 strains. We show that by use of recombinant gp120s, some degree of regulation of TLR7-triggered pDCs is observed. On inactivated HIV-1 strains, a higher strain-specific mannosylation of gp120 negatively correlated with the amount of IFN-α produced. However, high-mannose perturbed complete viral particles did not induce any change in the pDC phenotypes, therefore suggesting that this virulence mechanism is not important in the interplay between human pDCs and HIV-1.

5.3 Materials and Methods

Reagents. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: IIIB (CHO-derived), 96ZM651 (Sf9/baculovirus-derived) and BaL (HEK-293-derived). Aldrithiol (AT)-2 inactivated HIV-1 strains BaL and MN and corresponding microvesicle controls[217] were provided by the AIDS and Cancer Virus Program, SAIC Frederick Inc./National Cancer Institute supported with federal funds from the National Cancer Institute, National Institutes of Health, under contract HHSN261200800001E. R848 and CpG ODN2216 were purchased from Invivogen. The following anti-human Abs were used in the studies: HLA-DR/v500 (clone# G46-6), CD86/v450 (2331(FUN-1)), PD-L1/PE-Cy7 (M1H1), CD80/PE (L307.4), MR/APC (19.2), αMR (19.2) (all from BD Biosciences), CCR7/APC-eFlour780 (3D12), (eBioscience), CD40/PerCP-Cy5.5 (5C3) (Biolegend), BDCA-2/FITC/APC (AC144) (Miltenyi).

Human blood samples. Human buffy coats were acquired from the blood bank at the Copenhagen University Hospital, Rigshospitalet (Copenhagen, Denmark) collected from anonymous healthy donors. Blood samples were handled in accordance with guidelines put forward in the "Transfusion Medicine Standards" by the Danish Society for Clinical Immunology (www.dski.dk). The buffy coats were obtained from 450 mL whole blood donations in citrate phosphate dextrose anticoagulant. The whole

blood was fractionated to separate the majority of erythrocytes and plasma. The buffy coat contains approximately 90% of the leukocytes from the 450 mL of blood, and was used within 5.5 hours upon blood draw.

Cell purification and culture. Untouched human pDCs were isolated using the following three-step procedure: PBMCs were isolated from human buffy coats by Ficoll-Paque (GE Healthcare). The PBMCs were enriched for peripheral blood DCs by the Dynal human DC enrichment kit (Invitrogen). Enriched peripheral blood DCs were purified for pDCs using the EasySep human pDC enrichment kit (Stemcell Technologies). Purities ranged from 95-98% assessed by BDCA-2 expression. Cells were cultured in RPMI 1640 containing 2% (v/v) L-glutamine, 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES (all from Lonza), and 50 μM β-mercaptoethanol (Invitrogen). Cells were kept in a humidified 37°C, 5% CO_2 incubator. Monocyte-derived DCs were generated from CD14+ MACS-purified (Miltenyi) monocytes in culture medium supplied with 20 ng/mL recombinant human GM-CSF and 30 ng/mL recombinant human IL-4 (both CellGenix). Monocyte cultures were supplied with fresh GM-CSF and IL-4 on day 3, and non-adherent immature DCs were harvested on day 6. pDCs were stimulated with normalized concentrations of HIV-1 either according to p24 capsid concentration or according to multiplicity of infection (MOI). R848 was added at 2.5 μg/mL, CpG ODN2216 at 5 μM, and blocking αMR Ab at 5 μg/mL (added 30 min prior to stimulations).

HIV-1 culture. The following HIV-1 strains were used in these studies: BaL (R5), MN (X4), BX08 (R5), and NL4.3 (X4). HIV-1 strains were grown in donor PBMCs (Rigshospitalet) in RPMI 1640 containing 10% FBS, 1% penicillin/streptomycin and supplemented with recombinant human IL-2 (10 IU/mL) (Roche). Virus proliferation was measured by p24 ELISA[218] and at peak viral concentration cell free virus supernatants were harvested. Aliquots were stored at -80°C in order to limit differences between assays.

p24 ELISA. p24 concentration in HIV-1 preparations was measured using an in-house sandwich ELISA[218] (Statens Serum Institut, Copenhagen, Denmark). The p24 values obtained using this ELISA corresponded to the values determined for AT-2

HIV-1 strains by HPLC at the AIDS and Cancer Virus Program, SAIC Frederick, Inc./National Cancer Institute, Frederick.

Copy-number determination. Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen). Purified RNA and HIV-1 RNA standards were quantified by quantitative real time reverse transcription PCR using Superscript III system with Platinum Taq DNA Polymerase (Invitrogen). Primers and probes are described elsewhere.[219,220] Amplification was analyzed on an Mx3005P QPCR system (Agilent Technologies).

IFN-α2a ELISA. Supernatants from pDCs stimulated with viable HIV-1 were inactivated using Triton X-100 (Sigma Aldrich) at a final concentration of 1%. IFN-α2a was measured using a human tissue culture kit on a Sector Imager 2400 (both Meso Scale Discovery).

Flow cytometry. Flow cytometry was performed according to standard procedures using a FACSCanto II or a LSR II (both BD Biosciences). The wash and staining buffer consisted of PBS with 1% FBS and 0.1% Na-azid (Sigma-Aldrich). Before staining, the cells were incubated for 10 minutes in staining buffer with 2% allogeneic human AB plasma (Rigshospitalet) to limit non-specific binding of Abs. pDCs stimulated with viable HIV-1 were fixed in 2% paraformaldehyde (Polysciences) prior to acquisition of data. Viability of pDCs was measured using a LIVE/DEAD fixable dead cell stain kit with a near infrared fluorescent reactive dye (Invitrogen). FACS data was analyzed using FlowJo (Treestar).

Western Blot. HIV-1 preparations were lysed in RIPA buffer containing a protease-inhibitor cocktail (both Cell Signaling Technology). Proteins were loaded normalized according to p24 capsid concentration and separated under reducing conditions by SDS-PAGE in MOPS buffer (Invitrogen). Immobilon-P PVDF membrane (Millipore) was blocked for 1 hour at room temperature in Pierce Superblock with tween-20. Primary gp120 Ab (Abcam cat# ab21179) was incubated overnight at 4°C (1:2000 dilution in superblock), and secondary Ab-HRP (Abcam cat# ab6741) was incubated for 45 min at room temperature on rocker (1:20,000 dilution in superblock). HRP-conjugated high-mannose-specific lectin from *G. nivalis* (EY Laboratories) was incu-

bated overnight at 4°C (1:20,000 dilution in superblock). Protein/mannose bands were visualized using an ECL+ kit (GE Healthcare) and a G:BOX CCD camera (Syngene). Intensity of bands was determined by Genepix Pro software (Molecular Devices).

Enzymatic removal of high-mannose from HIV-1 strains. AT-2 inactivated and viable HIV-1 strains were mixed with 5X Endoglycosidase (Endo) H reaction buffer (250 mM sodium phosphate buffer, pH 5.5, Sigma Aldrich) and added either Endo H to a final concentration of 0.2 units/mL or vehicle control consisting of 20 mM Tris HCl, pH 7.5, with 25 mM NaCl and incubated at 37°C for 1 hour. Initially a time study was conducted to determine the optimal incubation time for the enzyme (Fig. S1). Endo H and free mannose-units were subsequently removed using Amicon ultra 0.5 centrifugal units with a 50 kDa cut-off (Millipore). After processing p24 concentration was re-assayed.

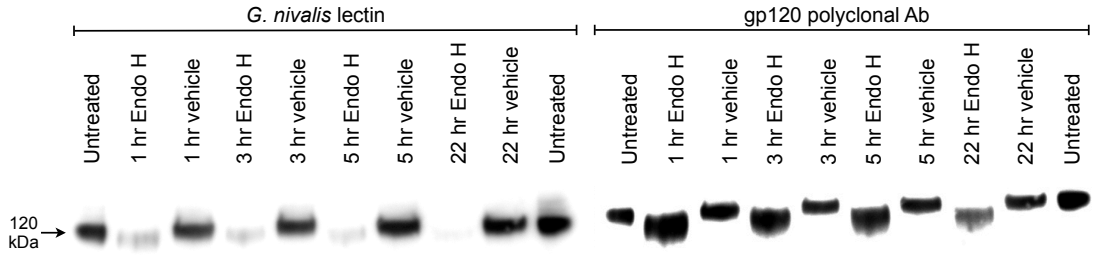

Figure S1: Endoglycosidase H time-course. AT-2 (aldrithiol-2) inactivated HIV-1 BaL was treated with Endoglycosidase H (Endo H) or vehicle control for the indicated times. Afterwards preparations were lysed and loaded onto a SDS-PAGE gel. Proteins were separated under reducing conditions and blotted onto a PVDF membrane. A polyclonal gp120-Ab (gp120) was used to determine the gp120 levels, and *G. nivalis* lectin to detect the presence of high-mannose structures on gp120.

Statistics. Statistical analysis was done by a Mann-Whitney, or a Wilcoxon matched-pairs signed rank test for comparison of two variables, and by a Kruskal-Wallis test with a Dunns' post-hoc test for comparison of more than two variables. Analysis was done using Prism 5 (Graphpad Software).

5.4 Results

HIV-1 recombinant gp120s diversely modulate pDC immunity

Because of recent reports describing that the glycosylation pattern on recombinant gp120 varies according to the expression system (Fig. 1A),[159] we decided to re-evaluate the conclusions in a former report describing a lack of crosstalk between ssRNA-triggered TLR7 and HIV-1 gp120.[147] As the concentration of monomeric gp120 may need to be very high in order to effectively trigger the receptors that natural trimeric gp120-gp41 complexes interact with (Fig. 1B), we titrated a range of different gp120s from 10-100 μg/mL added together with the TLR7-triggering ligand R848. In contrast to the former report,[147] one of the three gp120s tested induced a strong modulation of TLR7-induced IFN-α production when added at 100 μg/mL (96ZM651, Fig. 1C and E). 96ZM651 HIV-1 gp120 lead to approximately 80% reduction in the ssRNA (R848)-induced IFN-α production, while IIIB and BaL gp120 did not modify the R848-induced phenotype. None of the tested gp120s resulted in major regulation of a range of pDC surface markers (Fig. 1D and E). Most notably were a decrease in the co-stimulatory molecule CD40 by the IFN-α modifying gp120 (96ZM651), and an increase in another co-stimulatory molecule CD86 for the other two gp120s (Fig. 1E). The antigen-presenting molecule HLA-DR, the lymph node homing receptor CCR7,[187] and PD-L1, which has been associated with T cell exhaustion,[221] were only minimally up- and down-regulated by the three gp120s (Fig. 1E). It has previously been reported that MR is not expressed on pDCs, but here we observed that it was upregulated upon TLR7-triggering (Fig. 1F), thereby suggesting a possibly role for MR in high-mannose-induced modification of IFN-α production in pDCs. In comparison to the classically MR-expressing monocyte-derived DCs, the level of expression on pDCs was, however, on average 5.1-fold lower. DC-SIGN, another mannose-binding receptor, was not induced on activated pDCs (data not shown).

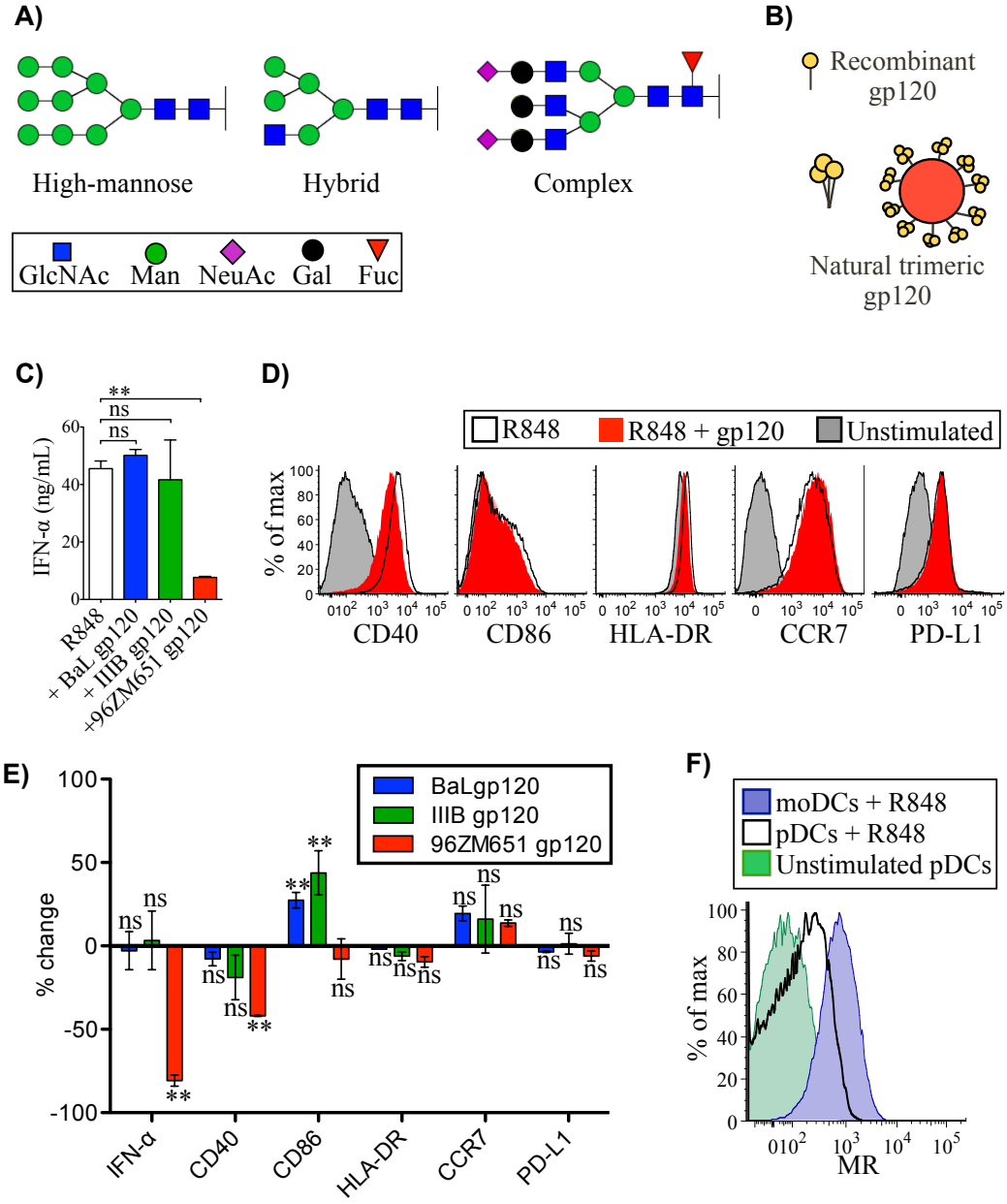

Figure 1: Phenotypic modulation of TLR7-triggered human pDCs by recombinant HIV-1 gp120s. A) Schematic representation of different glycosylation patterns found on recombinant gp120.[159] B) Schematic representation of the difference between monomeric recombinant gp120 and natural trimeric gp120 held together by non-covalent attachment to three transmembrane gp41 units.[222] C-E) Human pDCs were stimulated for 16 hours with R848 alone (TLR7-triggering ssRNA analogue) or in combination with three recombinant gp120s, derived from different HIV-1 strains (BaL, 96ZM651 and IIIB). After 16 hours, pDCs were analyzed for IFN-α production by ELISA (C and E), and surface marker expression by flow cytometry (D-E). C-D shows representative data from one donor, and E shows the average of all donors (n = 3-9, error bars are based on SD). E displays the % change induced by the three gp120s when added simultaneously with R848 as compared to R848 single stimulation. F) Representative flow cytometry histograms for Mannose Receptor (MR) expression on unstimulated and R848-activated pDCs compared to monocyte-derived DCs (moDCs) (n = 10).

Statistical analysis was done by a Mann-Whitney test (in C), and a Wilcoxon matched-pairs signed rank test (in E), **p<0.01. GlcNAc: N-Acetylglucosamine, Man: mannose, NeuAc: N-acetylneuraminic acid, Gal: galactose, Fuc: fucose.

HIV-1 gp120 mannosylation inversely correlates with IFN-α production by human pDCs

Given the results showing that pDCs can be modulated by at least one specific gp120, we decided to test whether this could be replicated when using complete HIV-1 particles. The mannosylation pattern of attenuated HIV-1 strains was analyzed by lectin-specific Western blot (Fig. 2A-B, where GNL indicates the degree of mannose on gp120). For co-culture studies with pDCs, two different HIV-1 strains with very different degree of mannosylation were employed (BaL and MN) (Fig. 2A-B). Upon stimulation of pDCs with these two HIV-1 strains, it was apparent that the HIV-1 strain with less gp120 mannose (MN) was able to activate the pDCs more profoundly than the strain (BaL) displaying more gp120-mannose (Fig. 2C-D). The most notable difference was seen at the level of IFN-α production, with an average 2.9-fold higher IFN-α production for MN (Fig. 2C). Small differences between MN and BaL were also seen in the level of expression of pDC activation markers (Fig. 2D). Notably, PD-L1 and CCR7 were expressed at a 1.3-fold and 1.8-fold higher level, respectively, upon activation with MN (less mannosylated) as compared to BaL. Likewise, MN also induced slightly higher levels of expression of the co-stimulatory molecules CD80, CD86 and CD40 than the HIV-1 strain with most mannose, while HLA-DR was upregulated to the same level by both strains (Fig. 2D).

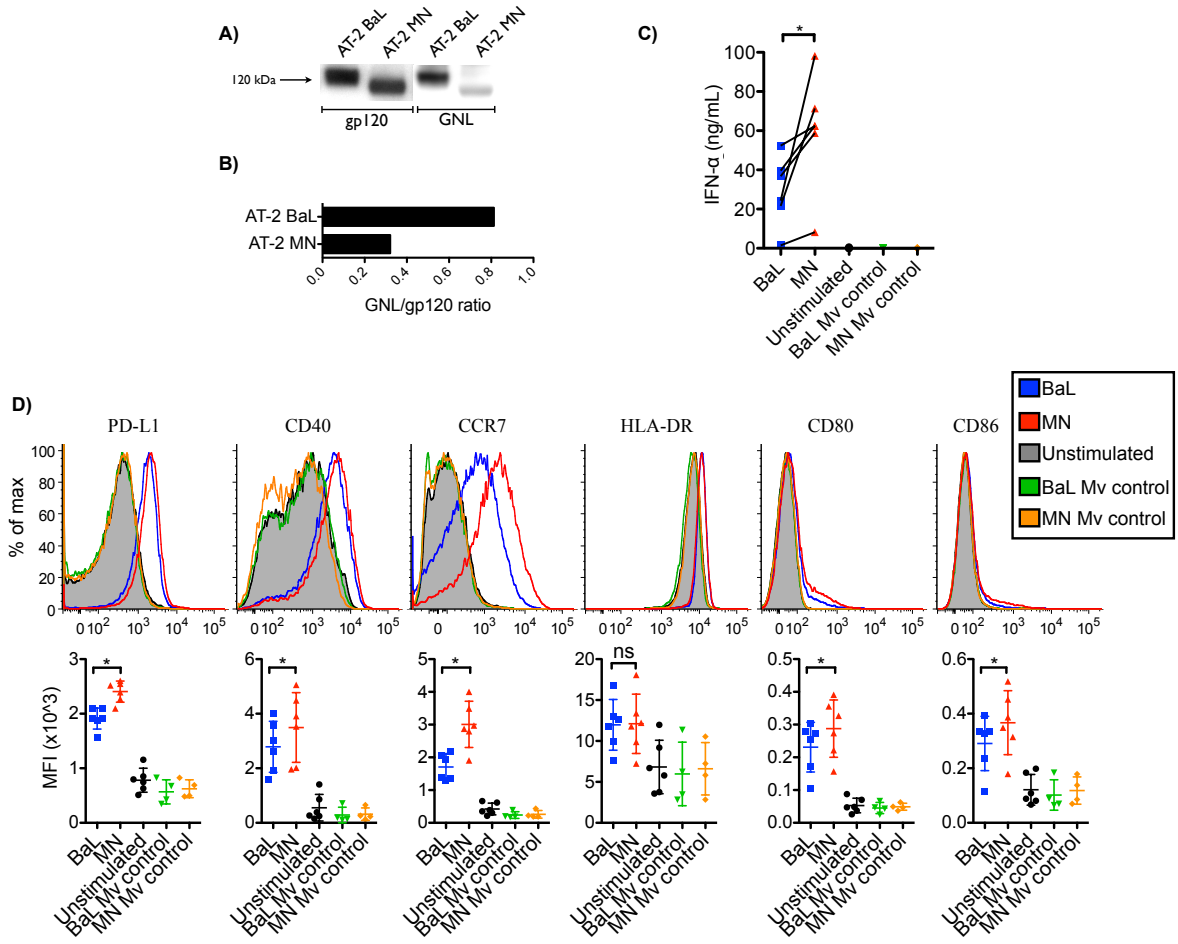

Figure 2: Maturation of pDCs with attenuated HIV-1 strains that are differentially mannosylated. A-B) Western blot analysis of AT-2 inactivated HIV-1 strains. gp120 was used as loading control, and mannosylation levels were visualized by the mannose specific lectin *G. nivalis* lectin (GNL). B shows the ratio between intensity of GNL and gp120 bands and is representative for 2 independent experiments. C-D) The phenotype of HIV-1 stimulated pDCs as measured by ELISA (C) and flow cytometry (D). Microvesicle controls (Mv) were included as a control for contaminating cellular proteins.[217] Statistical analysis was done by a Wilcoxon matched-pairs signed rank test, *p<0.05, **p<0.01, ***p<0.001. Error bars represent SD.

Perturbation of high-mannose on attenuated HIV-1 does not alter the pDC phenotype

To directly examine if the degree of high-mannose on HIV-1 gp120 plays a part in the level of IFN-α production in human pDCs, we enzymatically treated the HIV-1 strains to remove the high-mannose. A schematic representation of the cleavage site for the enzyme used to cleave of high mannose, Endo H, can be seen in Fig. 3A. Subsequently we tested the removal by lectin-specific Western blot (Fig. 3B-C). After Endo H treatment there was a visible perturbation of the high-mannose, seen by the lower

intensity of the GNL band after treatment, and by the band shift of the gp120 after mannose removal (Fig. 3B-C). The influence of high-mannose perturbation of gp120 on the HIV-1 strains was determined based on regulation of the functional phenotype in pDCs (Fig. 3D-E). Surprisingly, we noticed that there was no differences in the phenotypes, indicating that the gp120-mannose might not play the role we assumed, and which it has also been attributed in the literature.[147,148] This suggested that MR might not be involved in gp120-high mannose-mediated suppression of IFN-α production in pDCs, although the level of regulation of MR by ssRNA (Fig. 1F) and HIV-1 (Fig. 4A) infers a possible role. To further examine the involvement of MR, we therefore tested if blocking of the MR would affect IFN-α production upon ssRNA/HIV-1 stimulation. Blocking the MR resulted in no significant differences in the pDC response (Fig. 4B).

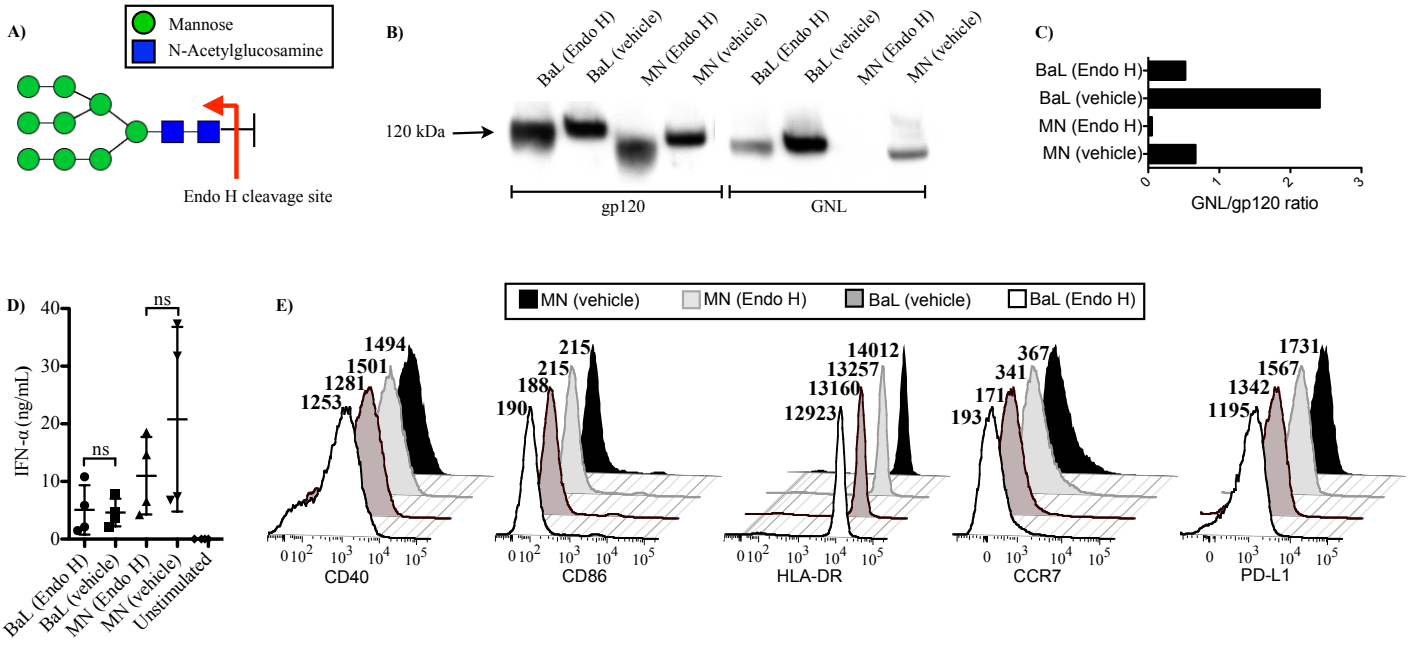

Figure 3: Removal of high mannose on AT-2 HIV-1 strains and the effect on pDC phenotype. A) Schematic representation of the cleavage site for Endoglycosidase H (Endo H). B-C) AT-2 inactivated HIV-1 strains were treated with Endo H or vehicle control, followed by removal of freed high-mannose and remainder of enzyme by spin columns. Western blot analysis was used to verify efficiency of the procedure. The ratio between intensity of GNL and gp120 bands were calculated and plotted in C), and are representative for 2 independent experiments. D-E) The phenotype of pDCs stimulated with high-mannose perturbed HIV-1 strains for 16 hours. IFN-α production was determined by ELISA and surface markers by flow cytometry. Flow cytometry histograms in E are representative for all donors. The

value next to the histogram is the MFI value for the different surface markers. Each dot represents the mean of duplicates from one donor (n = 4, error bars = SD). Differences between high-mannose perturbed HIV-1 strains and unperturbed control strains were tested using a Wilcoxon matched-pairs signed rank test.

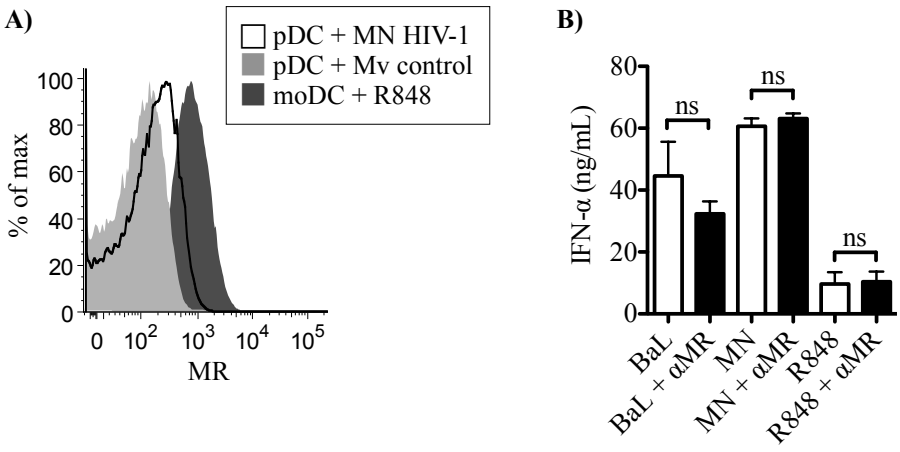

Figure 4: HIV-1 mediated upregulation of MR in pDCs and the effect of MR blocking. A) pDCs were stimulated with AT-2 inactivated HIV-1 strains or microvesicle (Mv) controls for 16 hours, and analyzed for Mannose Receptor (MR) expression by flow cytometry. Monocyte-derived DCs (moDCs) stimulated with R848 was added as a positive control. B) pDCs were pre-incubated with anti-MR Ab or vehicle control for 30 minutes prior to addition of HIV-1 or R848 for 16 hours. IFN-α production was subsequently analyzed by ELISA. Statistical analysis was done by a Mann-Whitney test, and error bars are SD. Data represents 2-6 donors.

Viable HIV-1 strains induce variable pDC responses, but are unaffected by gp120 high-mannose

Before finally excluding high-mannose from playing a role in pDC immunity, we sought to analyze if we could replicate the results obtained for attenuated HIV-1 strains with viable HIV-1 strains. The mannose was removed on 4 different viable HIV-1 strains: BaL, MN, BX08 and NL4-3 (Fig. 5A), and subsequently used to stimulate pDCs. Intriguingly the pDCs responded very differently to the 4 strains. Two of the strains (BaL and BX08) induced a dose-dependent necrosis in pDCs, having completely eradicated the pDCs at MOI 1 (Fig. 5B) irrespectively of the presence of gp120-mannose or not. The other two HIV-1 strains (MN and NL4-3) did not propagate more necrosis than unstimulated cells, and NL4-3 even increased the survival of pDCs. MN and NL4-3 upregulated surface markers on pDCs in a gp120-mannose independent manner (Fig. 5D), but surprisingly, NL4-3 did not mediate IFN-

α production even at a very high MOI (MOI 2,500) (Fig. 5C). The IFN-α production induced by viable MN was not modified by gp120-mannose perturbation (Fig. 5C), confirming our findings from gp120-mannose removal of the attenuated HIV-1 strains.

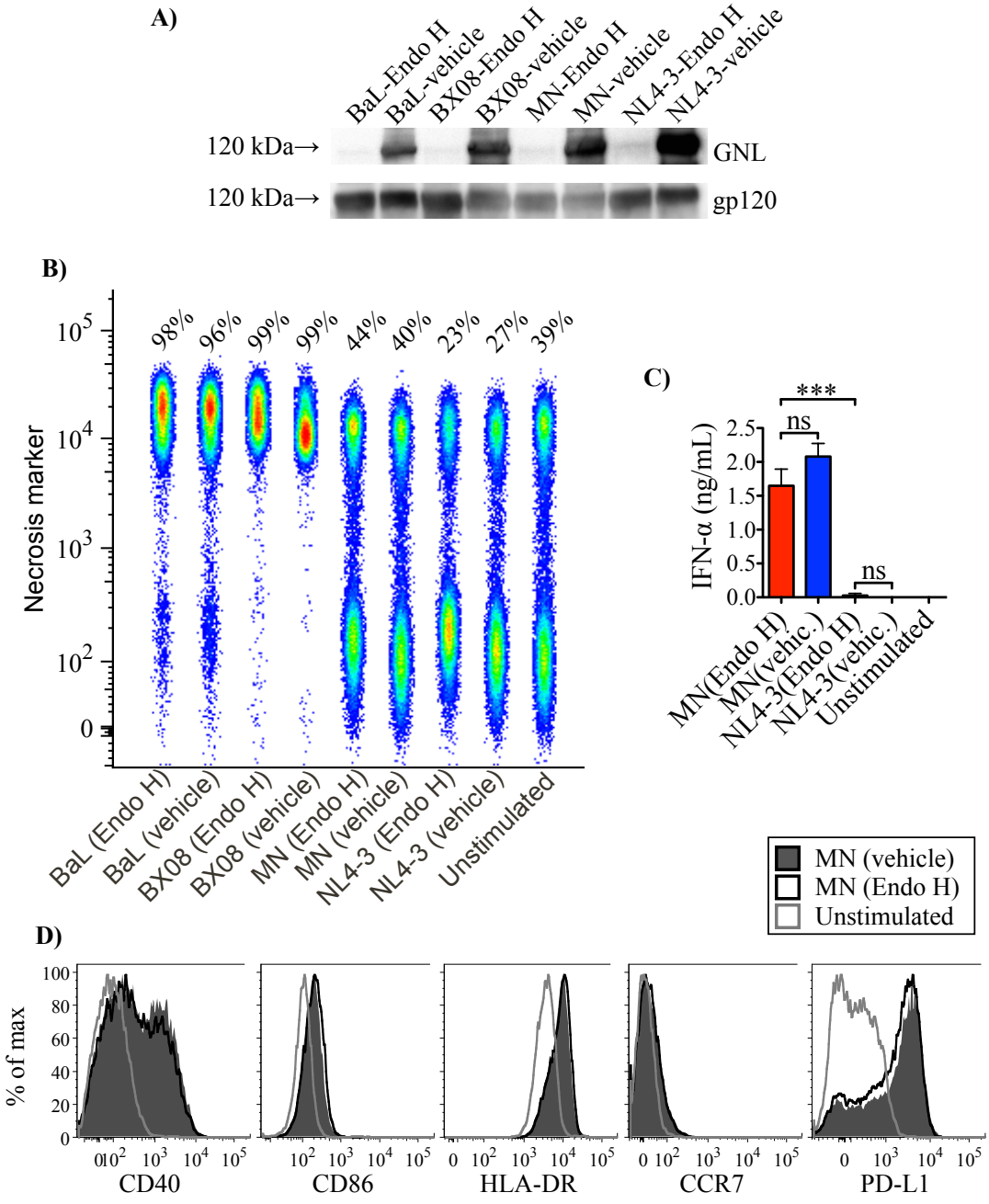

Figure 5: pDC response patterns to viable HIV-1 and effect of mannose perturbation. A) Western blot analysis of mannosylation degree on viable HIV-1 strains with and without Endo H-treatment. B-D) Phenotype of pDCs stimulated with high-mannose-perturbed (Endo H) and unperturbed (vehicle) viable HIV-1 strains. B) The percentage of necrotic pDCs (brightest population) was measured using a viability dye (near infrared (nIR) LIVE/DEAD stain). The percentage of cells falling within the dead

cell gate (not shown) is given above each dot plot. C) IFN-α production as measured by ELISA. D) Representative flow cytometry histograms from one donor. The data is representative for 3-7 donors. Statistical analysis was done by a Kruskal-Wallis test with a Dunns' post-hoc test, and error bars are SD, ***$p<0.001$.

High-mannose perturbed attenuated HIV-1 strains does not modulate a TLR-triggered pDC phenotype

Since we initially found that one recombinant gp120 were able to modify IFN-α production in TLR7-triggered pDCs, and others have shown the same for TLR9-triggered pDCs,[147] we finally tested whether the removal of high-mannose could affect these TLR-responses (Fig. 6). Remarkably, comparisons of the effects of high-mannose containing, and non-containing inactivated HIV-1 on TLR7 or TLR9-triggered pDC responses revealed that HIV-1 did not induce any change at all of the TLR-induced response; not even additive.

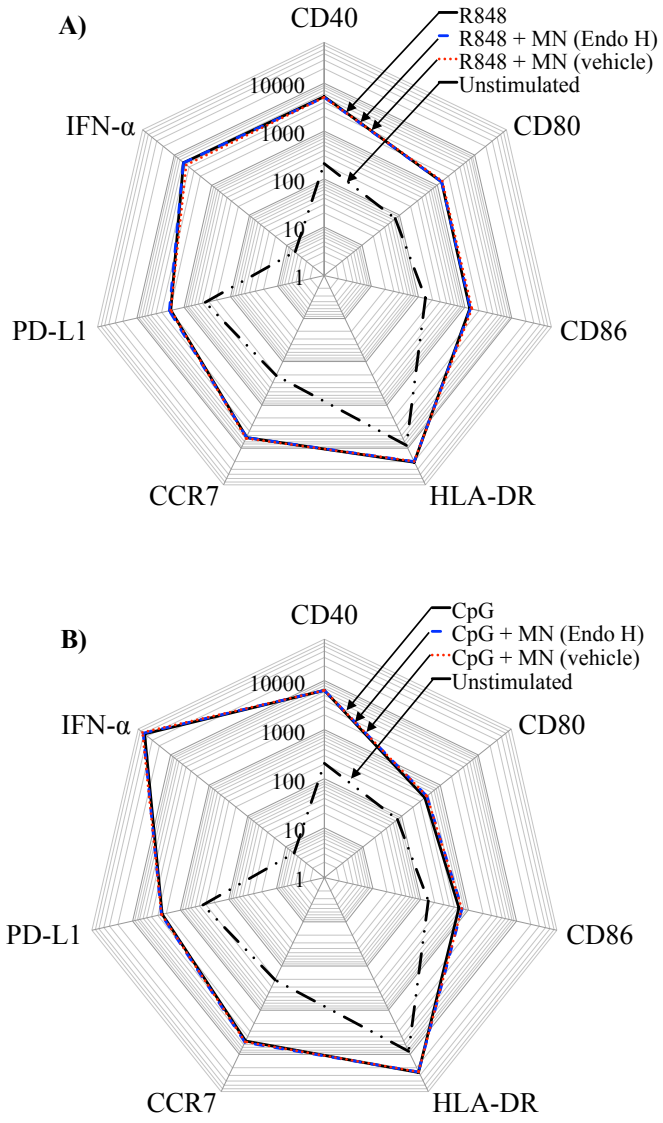

Figure 6: The effect of high-mannose perturbed HIV-1 strains on the phenotype of TLR-activated pDCs. Human pDCs were stimulated with R848 or CpG (TLR7 and TLR9 ligand, respectively) alone or together with high-mannose perturbed inactivated HIV-1 strains (Endo H) or unperturbed control strains (vehicle) for 16 hours. Afterwards pDCs were analyzed for surface marker expression by flow cytometry and IFN-α production by ELISA. The fold change in phenotype that was mediated by addition of HIV-1 strains to TLR7 (A) and TLR9 (B) activated pDCs is illustrated in the spider-web plots. The numbers on the y-axis denominates the fold change. Fold change was calculated for each donor separately and the average plotted on the graphs. Data are representative of two independent experiments.

5.5 Discussion

HIV-1 gp120 high-mannose has been shown to modify immune responses in mDCs, but the role on pDC immunity has not been thoroughly investigated. Former experi-

mental systems have consisted of recombinant gp120, which may not be glycosylated similarly to *in vivo* viral particles. Here we have used perturbed complete viral particles to demonstrate that HIV-1 gp120 high-mannose does not modulate immunity in human pDCs.

Similar to a previous study in monocyte-derived DCs,[149] we observed various regulatory potentials of different recombinant gp120s. Only one of three gp120s gave a significant decrease in IFN-α production, and only when added in a very high concentration of 100 μg/mL. When using complete viral particles mannosylated at different levels, it appeared that the mannosylation of the HIV-1 strains correlated inversely with the amount of IFN-α produced. However, upon perturbation of the mannosylation on each strain it was evident that the difference in IFN-α was independent on the level of gp120-mannosylation. Instead the varying levels of IFN-α in pDCs exposed to inactivated MN and BaL might be explained by the difference in tropism between the two HIV-1 strains. In our studies, we have used two CCR5 (R5)-tropic HIV-1 strains: BaL and BX08, and two X4 strains: MN and NL4-3. Collectively, the R5 strains induced more necrosis and less IFN-α compared to the X4 strains. One explanation for the difference might be found in the route of entrance used by the different strains. HIV-1 is reported to enter immune cells either via direct fusion with the plasma membrane or by receptor-mediated endocytosis.[141] Fusion is the process that may lead to effective infection and takes place when HIV-1 interacts with CD4 and a co-receptor on the host immune cell. Receptor-mediated endocytosis on the other hand leads to degradation of the virus inside acidified endolysosomes. In a report seeking out whether HIV-1 used endocytosis or fusion for the activation of pDCs, it was found that the employed X4 strain almost exclusively used endocytosis.[141] In another study focusing on infection, it was shown that pDCs were more susceptible to infection by R5 viruses compared to X4 viruses,[223] thus suggesting that R5 viruses are more likely to enter pDCs via fusion. To complicate matters more it was also shown that after endocytosis, and dependent on which endosomal compartment TLR7 or TLR9 are triggered in, pDCs either produce type I IFNs (early endosomal compartment) or increase co-stimulatory molecule expression (CD40, CD80, CD86) and produce inflammatory cytokines (late endosomal compartments).[224] These data may explain why one of our viable X4 strains (NL4-3) did not induce IFN-α while being efficient in mediating up-regulation of co-stimulatory molecules on pDCs.

When reviewing published studies in detail there are some noticeable differences in the pDC responses to R5 and X4 HIV-1 strains that appear similar to our present findings. For instance, pDCs stimulated with the same amount of two HIV-1 strains as measured by p24 capsid, (MN vs. JRCSF[140] and BaL vs. IIIB[225]) showed a reduced production of IFN-α for the R5 as opposed to the X4 strains. In contrast to the R5 strains used in our study, these R5 strains did not induce necrosis in the pDCs. The necrosis inducing effect may therefore depend on the given R5 strain, or by the culture system used to produce the virus. When HIV-1 is grown in culture it is bound to mutate, and perhaps some of these HIV-1 preparations have lost the ability to kill the pDCs. In fact, because of this dichotomy we cultivated one of our R5 strains (BX08) again and this preparation somehow lost the ability to kill the pDCs. But it did not acquire the ability to induce IFN-α, not even when added at MOI 25. To make sure that the medium composition was not influencing the killing of pDCs, we up-concentrated the initial HIV-1 preparation and added fresh medium instead, but again we observed killing of 99% of the pDCs at MOI 1, hence implying that the R5-induced cellular atrophy in pDCs was due to the virus *per se*. It has been shown that after an HIV-1 infection, pDCs disappear from peripheral circulation,[226–228] reflecting that they might migrate to the draining lymph nodes[229,230] or the spleen.[231] Our present findings indicate that the pDC disappearance also could be due to R5-induced necrosis of pDCs at site of infection, possibly influencing systemic recruitment of pDCs. Small induction of pDC cell death has been demonstrated previously upon interaction between a cell line chronically infected with IIIB (X4) HIV-1 and PBMCs.[154] The prior study did, however, not include R5 viruses, and our data are therefore the first to demonstrate an R5-mediated eradication of pDCs. As this observation may serve important in relation to *in vivo* combat of HIV-1 at initial stages of infection, we are currently undertaking analysis to study the mechanism behind the HIV-1-induced necrosis in greater details.

In conclusion, our data exclude a role for high-mannose-mediated immune suppression of pDC by HIV-1. Explicit comparisons of viable R5 and X4 HIV-1 strains suggest that R5 strains might impede pDC immunity by inducing cellular death in pDCs, thus expanding the avenue of evasion mechanisms exploited by HIV-1 on innate immunity.

6 Final discussion

Mtb and HIV-1 have both evolved excellent ways to evade the immune system, in order to avoid clearance. Common for these pathogens is their early interaction with DCs, which, in a manner similar to the Butterfly Effect, could have major impact on how the immune response develops at later stages. A tiny wicked signal at the initial interaction could potentially propagate into a huge impact on the final outcome. The current Ph.D. thesis have demonstrated that this may be the case for both HIV-1 and Mtb. R5 HIV-1 strains usually found early in infection could be eliminating the pDCs, thereby inhibiting proper activation of mDCs, which might have a detrimental role in the establishment of the founder HIV-1 population. Likewise Mtb may evade IL-12p70-mediated Th1 induction, by exploiting the plasticity of moDCs, and by inducing a prototypic extracellular response against this intracellular bacterium. If the signals delivered by DCs are either lacking (HIV-1 example) or misdirecting (Mtb example), the remaining immune cells will not be able to respond correctly in order to clear the infection.

The motivation behind this thesis was to generate a better understanding of the interaction between human DCs and the latency-inducing pathogens: Mtb and HIV-1. Specifically the focus has been to elucidate how Mtb avoids IL-12p70 production in moDCs, and if HIV-1 gp120 high-mannose modulates the phenotype of pDCs. Prior to the initiation of this work, a paradigm was emerging on how these pathogens could avoid clearance from our bodies, by virtue of a glycan-mediated dampening of the ensuing immune response.[148] Therefore, a special focus was given to this type of immune evasion strategy in both cell types. In contrast to what was expected, major glycan-mediated changes were not observed. pDCs appeared to be unaffected by HIV-1 mannosylation, and moDCs were only modulated to a small extend by the Mtb-expressed glycolipid ManLAM. However, even though the glycan-mediated modulations in moDCs were smaller than expected, they are most likely one of many immune evasion strategies Mtb employs to avoid clearance from the body. After all, Mtb has been around for ages with the first observations dating back to 5000 years B.C.,[232] therefore enabling Mtb to develop many different immune evasion strategies over time.

MoDCs has been used for many years as an *in vitro* analogue to test response patterns of mDCs found *in vivo*. However, recently it was demonstrated that moDCs may play a role *in vivo*,[115,176] and in particular interest for Mtb-infections, they have been shown to migrate into the lungs of mice.[115] Furthermore, both CD1a+ and CD1a- mDCs has been described in the human lungs.[111,114,174,175] Testing if moDCs have any physiologic relevance in humans is, however, almost impossible, which should be kept in mind when interpreting the data generated using this system. One of the interesting observations made during this thesis work is the diverse ability of different donors to generate CD1a-hi moDCs. In chapter 2, it was described how the percentage of CD1a-hi moDCs generated using the same exact culture conditions varied between donors in a normal-distributed manner. If these moDCs have an *in vivo* relevance in humans, this may be able to explain why different individuals have very different response patterns to mycobacteria. The capability to develop protective immunity against mycobacteria is irregularly distributed among human beings. Only 10% of Mtb infected individuals develop active TB, whereas the main population of infected people carry a latent infection. The number of people able to resist Mtb infection is, naturally, not known. From cases of *M. leprae* infected individuals, it is known that this mycobacteria can cause two types of diseases in different persons; i) tuberculoid leprosy characterized by a IL-12p70-induced Th1 response, and ii) lepromateous leprosy characterized by a Th2 response.[198,199] Of these, the Th1-mediated response type has shown to be the most effective immune response against *M. leprae*. The importance of the Th1-response in keeping mycobacterial infections under control can also be seen from patients with Mendelian susceptibility to mycobacterial disease.[68] The mutations possessed by these patients in one of the genes: *IFNGR1, IFNGR2, STAT1, IL12B, and IL12BR1* are very rare and would not be able to explain why many mycobacteria-infected individuals do not develop a protective Th1 response. Most likely more subtle common genetic variations contributes to mycobacteria susceptibility,[233] which may be apparent if examining more carefully the phenotypes of the immune cells interacting with mycobacteria. By re-assaying the data presented in chapter 2, and comparing the phenotype of the entire pool of moDCs from the two extremes in CD1a-expression, two types of immune responses may be presented (Fig. 6.1). It could therefore be speculated that a patient in the lower CD1a-hi range would develop active TB and lepromateous leprosy more easily, because of the diminished production of IL-12 family cytokines. Likewise, a patient in the high range of CD1a-hi DCs

might be able to either clear an Mtb-infection or develop tuberculoid leprosy. If this holds true, given that the number of CD1a-hi DCs follows a Gaussian distribution, you might generally expect that more than 10% of Mtb-infected patients would develop active TB. At the current stage it is, however, impossible to say how many CD1a-hi DCs are needed to develop protective immunity. But based on the available epidemiological TB data it seems plausible that a low number of CD1a-hi DCs are needed in the lung tissue to prevent active disease, while larger numbers are required to avert latent Mtb infection.

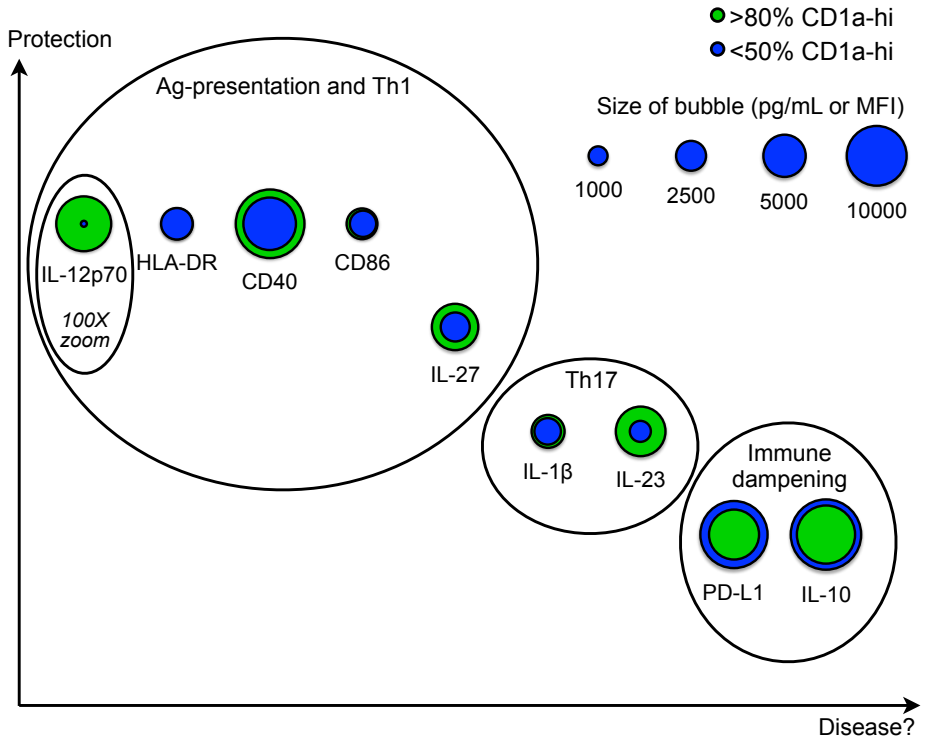

Figure 6.1: Overview of the heterogeneous immune responses against Mtb. moDCs were stimulated with Mtb for 24 hours and analyzed for surface marker expression by flow cytometry, and secreted cytokines by ELISA. The percentage of CD1a-hi DCs were noted and used to separate the DCs and thereby the response patterns into two groups based on <50% CD1a-hi expressing DCs (n = 7, blue bubbles) or >80% CD1a-hi expressing (n = 6, green bubbles). Afterwards the collected MFI for all DCs were extracted and used for the visualization. The average of all donors were used to calculate the bubble size, which represents MFI or pg/mL. One axis represents protection against Mtb (reflecting reported data in literature), while the other axis represents possible active disease progression.

Currently the immune response against Mtb is described as being dominated by Th1/Th17 cells without discriminating these responses. It can be speculated, that an

early Th17 response could mediate granuloma formation by recruiting the wrong type of immune cells (i.e. neutrophils), thereby shielding of infected macrophages from the right type of immune cells arriving later during infection. An early Th1 response might instead be able to eliminate all infected cells, and may therefore lead to eradication of the infection. Th1 responses are taking place during an Mtb-infection, but currently the time-frame for induction of this response is uncertain. In mice experiments employing three different mouse strains only one of the strains gave a robust Th1 response, and this response was not seen until 30 days after infection,[234] indicating that initially in infection the Th1 response may be absent. Indirect evidence for an initial Th17 response can also be seen from neutrophilic arrival at the site of infection very early after initiation.[235] If an early Th1 response were taking place, it would most likely be induced with help from γδT cells or other innate lymphocytes producing IFN-γ necessary to boost IL-12p70 in DCs. So far no one has demonstrated the presence of γδT cells in the lungs at steady state, but in monkeys small numbers have been found in the BALF two weeks after vaccination with BCG.[236] If the γδT cells are not present before infection, or if they do not come into contact with DCs in the lung, this important link for early Th1 development may be missing. The γδT cells could also produce IL-17A or IL-22 instead of IFN-γ and in this scenario, IL-12p70 production from DCs would not be boosted either, as suggested from the data presented in chapter 2.

An important point that can be learned from the studies on the interaction between HIV-1 and pDCs is to take into account the complexity of different HIV-1 strains when conducting research. In future studies on HIV-1's interaction with the immune system it would be advantageous to distinguish between HIV-1 strains found early in infection and the ones found in late stage-disease, in order to determine how HIV-1 generates the all-important founder population. Even though it has been determined that in the most cases the founder population originates from R5 strains,[124,125] it is still difficult to completely ensure that the specific HIV-1 strains isolated are the ones that actually initiate infection, due to the high mutation rate of HIV-1 strains. In the studies conducted in this thesis we used a strain that is considered an early HIV-1 strain. This strain (BX08) was isolated 8 months after seroconversion,[237] which means that, even though the strain is considered an early strain, in reality the strain would have

had plenty of time to mutate. However, HIV-1 strains isolated as early as possible are still the ones most likely to resemble the founder strain, and they currently provide the best alternative as a model system. To examine the hypothesis about R5-mediated killing of pDCs early in infection an *in vivo* study in monkeys could be conducted. In these experiments, the dose of HIV-1 should be so low that only certain strains can initiate infection, which could give a clue about which strains it is that are able to generate the founder population.

A different complexity that is also really hard to study is the kinases activated in DCs upon pathogen encounter. In this study a specific time point was selected for the kinome analysis, even though the optimal experiment would be to do a time-course from approximately 10 sec to 60 min, since phosphorylation and de-phosphorylation is taking place very rapidly. The reason why a time-course was not done in these studies was that the number of cells available was too low to assay more than one time point in one donor at a time due to sensitivity restraints in MS-based phosphoproteomics. Over time the sensitivity of MS-based phosphoproteomics will most likely get better, making it possible to conduct a time study. It will be interesting to repeat the Mtb kinome experiments at a later point.

A better understanding of how the immune system is diverted by HIV-1 and Mtb, may lead the way for new and better treatments. The most important effector molecule produced by pDCs is IFN-α, which by itself may be fulfilling the role of activating mDCs and other immune cells. Therefore IFN-α could be a treatment-possibility to induce immune activation early after HIV-1 infection. Of course it would be detrimental to the host to inject IFN-α systemically, and a more targeted approach is needed in order to make this feasible. It should be a strategy that induces the response almost immediately after infection, as it is currently believed that as soon as the founder HIV-1 population is there; it is too late for interventions. A targeted approach may also be useful for treating Mtb. The purpose here would be to target the alveolar DCs, or the monocytes that replenish the DC pool so they at immediate impact with Mtb would produce copies amounts of IL-12p70, and induce a more profound Th1 rather than Th17 response. The targeted strategy could aim at inhibiting some of the kinases

identified in this thesis to be uniquely activated by Mtb. Even if a successful strategy for diverting the immune response towards Th1 was found, the data from chapter 2 might indicate that only a certain amount of people would respond. If a person were pre-encoded to have a lot of CD1a-lo DCs incapable of producing IL-12p70, a Th1-driving drug would most likely not have a great effect. But these reflections need to be justified in future studies.

Given that moDCs are currently used for immunotherapy in various cancers, the differences in moDC response types between donors could have detrimental roles for the moDC preparations used in this setting. There is of course certain release criteria for moDCs used for clinical applications, for instance, one release criteria has been that >80% of the moDCs should express CD80 and CD86.[238] Our current data questions whether this is a proper release control, as can be seen from figure 6.1, CD86 does not vary much between donors from either side of the CD1a-expression range. One could therefore speculate that the diminished effectiveness of these vaccines could be due to injection of DCs with a tolerogenic function against the pulsed tumor-Ags. Clinical protocols use a higher concentration of IL-4 and GM-CSF than we did, and human plasma instead of FBS. These factors could affect the amount of CD1a-hi moDCs generated. We did test if increasing the concentration of IL-4 and GM-CSF would modulate the ratio of CD1a-hi moDCs in a couple of donors, and interestingly it did not. However, different recombinant cytokines could also affect the CD1a-development, and it was outside the scope of this thesis to fully test how these different protocols affect the moDC phenotype.

Even though the results presented in this thesis can help explain some of the interaction between our immune system and HIV-1 or Mtb, we are still far from understanding the grant complexity of the interactions taking place *in vivo*. Some of the unresolved questions waiting to be answered are listed here:

- Does Mtb take advantage of moDC plasticity *in vivo*?
- Can CD1a-distribution explain differences in responsiveness to mycobacteria *in vivo*?

- Are γδT cells, or perhaps innate lymphoid cells, inducing IFN-γ at the site of Mtb infection for early enhancement of the DC response *in vivo*?
- Does R5 HIV-1-induced pDC necrosis take place *in vivo*, and can this be the missing link to discrimination of HIV-1 tropism at the portal of entry?
- How does R5 HIV-1 induce necrosis in pDCs, and can this be exploited for vaccine construction?
- Could removal of CD1a-lo moDCs improve cancer immunotherapy?

In conclusion, the data presented in this thesis clearly demonstrates how important it is to study the initial interaction between the immune system and pathogens. Instead of letting HIV-1 and Mtb use the Butterfly Effect for their advantage, we should ourselves take advantage of this theory for treating these diseases. Making a drug that induces a small difference as soon as the pathogen is encountered, could divert the immune response into the correct direction and mean huge differences at the final stages. This could lead the way to eradication of one or both of these notorious pathogens: HIV-1 and Mtb, and result in a better life for a vast amount of people.

7 References

1. World Health Organization, UNICEF & UNAIDS *Global HIV/AIDS response progress report*. (2011).

2. World Health Organization Tuberculosis fact sheet # 104. (2010).at <http://www.who.int/mediacentre/factsheets/fs104/en/>

3. World Health Organization *Global tuberculosis control report*. (2011).

4. The Antiretroviral Therapy Cohort Collaboration. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet* **372**, 293-9 (2008).

5. Nielsen, M.H., Pedersen, F.S. & Kjems, J. Molecular strategies to inhibit HIV-1 replication. *Retrovirology* **2**, 10 (2005).

6. Oettinger, T., Jørgensen, M., Ladefoged, A., Hasløv, K. & Andersen, P. Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tuber. Lung. Dis.* **79**, 243-250 (1999).

7. Vandiviere, H.M., Dworski, M., Melvin, I.G., Watson, K.A. & Begley, J. Efficacy of bacillus Calmette-Guérin and isoniazid-resistant bacillus Calmette-Guérin with and without isoniazid chemoprophylaxis from day of vaccination. II. Field trial in man. *Am. Rev. Respir. Dis.* **108**, 301-13 (1973).

8. Baily, G.V. Tuberculosis prevention Trial, Madras. *Indian J. Med. Res.* **72 Suppl.**, 1-74 (1980).

9. Russell, D.G. Mycobacterium tuberculosis and the intimate discourse of a chronic infection. *Immunol. Rev.* **240**, 252-68 (2011).

10. Fischbach, M.A. & Walsh, C.T. Antibiotics for emerging pathogens. *Science* **325**, 1089-93 (2009).

11. United Nations *The millennium development goals report*. (2011).

12. World Health Organization Global tuberculosis database. (2012).at <http://www.who.int/tb/country/global_tb_database/en/index2.html>

13. Kaufmann, S.H.E. Future vaccination strategies against tuberculosis: thinking outside the box. *Immunity* **33**, 567-77 (2010).

14. Lorenz, E.N. Deterministic Nonperiodic Flow. *J. Atmos. Sci.* **20**, 130–141 (1963).

15. Haase, A.T. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* **464**, 217-223 (2010).

16. Winslow, G.M., Cooper, A., Reiley, W., Chatterjee, M. & Woodland, D.L. Early T-cell responses in tuberculosis immunity. *Immunol. Rev.* **225**, 284-99 (2008).

17. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245-52 (1998).

18. Trinchieri, G. & Sher, A. Cooperation of Toll-like receptor signals in innate immune defence. *Nat. Rev. Immunol.* **7**, 179-90 (2007).

19. Meyer-Wentrup, F. *et al*. Targeting DCIR on human plasmacytoid dendritic cells results in antigen presentation and inhibits IFN-alpha production. *Blood* **111**, 4245-4253 (2008).

20. Meyer-Wentrup, F. *et al*. DCIR is endocytosed into human dendritic cells and inhibits TLR8-mediated cytokine production. *J. Leukoc. Biol*. **85**, 518-25 (2009).

21. Dzionek, A. *et al*. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J. Exp. Med*. **194**, 1823-1834 (2001).

22. Geijtenbeek, T.B.H. *et al*. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med*. **197**, 7-17 (2003).

23. Caparrós, E. *et al*. DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. *Blood* **107**, 3950-8 (2006).

24. Chieppa, M. *et al*. Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J. Immunol*. **171**, 4552-4560 (2003).

25. Chen, C. *et al*. Dendritic-cell-associated C-type lectin 2 (DCAL-2) alters dendritic-cell maturation and cytokine production. *Blood* **107**, 1459-67 (2006).

26. Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M. & Puzo, G. Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor. *J. Immunol*. **166**, 7477-85 (2001).

27. Alvarez, Y. *et al*. Notch- and transducin-like enhancer of split (TLE)-dependent histone deacetylation explain interleukin 12 (IL-12) p70 inhibition by zymosan. *J. Biol. Chem*. **286**, 16583-95 (2011).

28. Gerosa, F. *et al*. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J. Exp. Med*. **205**, 1447-1461 (2008).

29. Collins, K.R., Quiñones-Mateu, M.E., Toossi, Z. & Arts, E.J. Impact of tuberculosis on HIV-1 replication, diversity, and disease progression. *AIDS Rev*. **4**, 165-76 (2002).

30. Lawn, S.D. AIDS in Africa: the impact of coinfections on the pathogenesis of HIV-1 infection. *J. Infect*. **48**, 1-12 (2004).

31. Ranjbar, S. *et al*. HIV-1 replication is differentially regulated by distinct clinical strains of Mycobacterium tuberculosis. *PloS one* **4**, e6116 (2009).

32. Toossi, Z. Virological and immunological impact of tuberculosis on human immunodeficiency virus type 1 disease. *J. Infect. Dis*. **188**, 1146-55 (2003).

33. Keane, J. *et al*. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N. Engl. J. Med*. **345**, 1098-104 (2001).

34. Ramakrishnan, L. Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol*. **12**, 352-366 (2012).

35. Tailleux, L. *et al*. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. *J. Exp. Med*. **197**, 121-127 (2003).

36. Jiao, X. *et al*. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. *J. Immunol* **168**, 1294-1301 (2002).

37. Wolf, A.J. *et al*. Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J. Immunol*. **179**, 2509-2519 (2007).

38. Desjardins, M., Huber, L.A., Parton, R.G. & Griffiths, G. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. *J. Cell. Biol*. **124**, 677-88 (1994).

39. Cohn, Z.A. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leucocytes and macrophages. *J. Exp. Med*. **117**, 27-42 (1963).

40. Russell, D.G. Who puts the tubercle in tuberculosis? *Nat. Rev. Microbiol*. **5**, 39-47 (2007).

41. Tailleux, L. *et al*. Constrained intracellular survival of Mycobacterium tuberculosis in human dendritic cells. *J. Immunol*. **170**, 1939-48 (2003).

42. Chackerian, A.A., Alt, J.M., Perera, T.V., Dascher, C.C. & Behar, S.M. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. *Infect. Immun*. **70**, 4501-9 (2002).

43. Torrelles, J.B., Azad, A.K. & Schlesinger, L.S. Fine discrimination in the recognition of individual species of phosphatidyl-myo-inositol mannosides from Mycobacterium tuberculosis by C-type lectin pattern recognition receptors. *J. Immunol*. **177**, 1805-16 (2006).

44. Schlesinger, L.S., Hull, S.R. & Kaufman, T.M. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of Mycobacterium tuberculosis to human macrophages. *J. Immunol*. **152**, 4070-9 (1994).

45. Harding, C.V. & Boom, W.H. Regulation of antigen presentation by Mycobacterium tuberculosis: a role for Toll-like receptors. *Nat. Rev. Microbiol*. **8**, 296-307 (2010).

46. Barber, G.N. Cytoplasmic DNA innate immune pathways. *Immunol. Rev*. **243**, 99-108 (2011).

47. Hemmi, H. *et al*. A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740-5 (2000).

48. Kadowaki, N. *et al*. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med*. **194**, 863-9 (2001).

49. Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. & Lanzavecchia, A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur. J. Immunol*. **31**, 3388-93 (2001).

50. Hoene, V., Peiser, M. & Wanner, R. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. *J. Leukoc. Biol*. **80**, 1328-36 (2006).

51. Abel, B. *et al*. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *J. Immunol*. **169**, 3155-62 (2002).

52. Reiling, N. *et al*. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. *J. Immunol*. **169**, 3480-4 (2002).

53. Uehori, J. *et al*. Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. *J. Immunol*. **174**, 7096-103 (2005).

54. Jung, I.D. *et al*. Enhanced efficacy of therapeutic cancer vaccines produced by co-treatment with Mycobacterium tuberculosis heparin-binding hemagglutinin, a novel TLR4 agonist. *Cancer Res*. **71**, 2858-70 (2011).

55. Henderson, R.A., Watkins, S.C. & Flynn, J.L. Activation of human dendritic cells following infection with Mycobacterium tuberculosis. *J. Immunol*. **159**, 635-643 (1997).

56. Zenaro, E., Donini, M. & Dusi, S. Induction of Th1/Th17 immune response by Mycobacterium tuberculosis: role of dectin-1, Mannose Receptor, and DC-SIGN. *J. Leukoc. Biol*. **86**, 1393-401 (2009).

57. Giacomini, E. *et al*. Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response. *J. Immunol*. **166**, 7033-7041 (2001).

58. Trinath, J., Maddur, M.S., Kaveri, S.V., Balaji, K.N. & Bayry, J. Mycobacterium tuberculosis promotes regulatory T-cell expansion via induction of programmed death-1 ligand 1 (PD-L1, CD274) on dendritic cells. *J. Infect. Dis*. **205**, 694-696 (2012).

59. Nathan, C.F., Murray, H., Wiebe, M. & Rubin, B. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J. Exp. Med*. **158**, 670-689 (1983).

60. Schreiber, R., Pace, J., Russell, S., Altman, A. & Katz, D. Macrophage-activating factor produced by a T cell hybridoma: physiochemical and biosynthetic resemblance to gamma-interferon. *J. Immunol*. **131**, 826-832 (1983).

61. Nathan, C.F. *et al*. Administration of recombinant interferon gamma to cancer patients enhances monocyte secretion of hydrogen peroxide. *Proc. Natl. Acad. Sci. USA* **82**, 8686-8690 (1985).

62. Kato, T., Hakamada, R., Yamane, H. & Nariuchi, H. Induction of IL-12 p40 messenger RNA expression and IL-12 production of macrophages via CD40-CD40 ligand interaction. *J. Immunol*. **20**, 3932-38 (1996).

63. Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R. & Melief, C.J. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480-3 (1998).

64. Husmann, L.A. & Bevan, M.J. Cooperation between helper T cells and cytotoxic T lymphocyte precursors. *Ann. N. Y. Acad. Sci*. **532**, 158-69 (1988).

65. Bennett, S.R., Carbone, F.R., Karamalis, F., Miller, J.F. & Heath, W.R. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J. Exp. Med*. **186**, 65-70 (1997).

66. Serbina, N.V., Liu, C.C., Scanga, C.A. & Flynn, J.L. CD8+ CTL from lungs of Mycobacterium tuberculosis-infected mice express perforin in vivo and lyse infected macrophages. *J. Immunol*. **165**, 353-63 (2000).

67. Heusel, J.W., Wesselschmidt, R.L., Shresta, S., Russell, J.H. & Ley, T.J. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. *Cell* **76**, 977-87 (1994).

68. Casanova, J.L. & Abel, L. Genetic dissection of immunity to mycobacteria: the human model. *Annu. Rev. Immunol*. **20**, 581-620 (2002).

69. Flynn, J.L. *et al*. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J. Exp. Med*. **178**, 2249-54 (1993).

70. Cooper, A.M. *et al*. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med*. **178**, 2243-7 (1993).

71. Hsieh, C.S. *et al*. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260**, 547-9 (1993).

72. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol*. **3**, 133-146 (2003).

73. Lucas, S., Ghilardi, N., Li, J. & de Sauvage, F.J. IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. USA* **100**, 15047-52 (2003).

74. Owaki, T. *et al*. A role for IL-27 in early regulation of Th1 differentiation. *J. Immunol*. **175**, 2191-200 (2005).

75. Demangel, C., Bertolino, P. & Britton, W.J. Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production. *Eur. J. Immunol*. **32**, 994-1002 (2002).

76. Redford, P.S. *et al*. Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur. J. Immunol*. **40**, 2200-10 (2010).

77. Ní Cheallaigh, C., Keane, J., Lavelle, E.C., Hope, J.C. & Harris, J. Autophagy in the immune response to tuberculosis: clinical perspectives. *Clin. Exp. Immunol*. **164**, 291-300 (2011).

78. Scriba, T.J. *et al*. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J. Immunol*. **180**, 1962-70 (2008).

79. Aujla, S.J. *et al*. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med*. **14**, 275-81 (2008).

80. Khader, S.A. *et al*. IL-23 compensates for the absence of IL-12p70 and is essential for the IL-17 response during tuberculosis but is dispensable for protection and antigen-specific IFN-gamma responses if IL-12p70 is available. *J. Immunol*. **175**, 788-795 (2005).

81. Khader, S.A. *et al*. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat. Immunol*. **8**, 369-77 (2007).

82. Evans, H.G., Suddason, T., Jackson, I., Taams, L.S. & Lord, G.M. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proc. Natl. Acad. Sci. USA* **104**, 17034-9 (2007).

83. Wilson, N.J. *et al*. Development, cytokine profile and function of human interleukin 17–producing helper T cells. *Nat. Immunol*. **8**, 950-957 (2007).

84. Acosta-Rodriguez, E.V., Napolitani, G., Lanzavecchia, A. & Sallusto, F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat. Immunol*. **8**, 942-9 (2007).

85. Chen, Z., Tato, C.M., Muul, L., Laurence, A. & O'Shea, J.J. Distinct regulation of interleukin-17 in human T helper lymphocytes. *Arthritis Rheum*. **56**, 2936-46 (2007).

86. Cosmi, L. *et al*. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J. Exp. Med*. **205**, 1903-16 (2008).

87. Santarlasci, V. *et al*. TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. *Eur. J. Immunol*. **39**, 207-15 (2009).

88. Marin, N.D. *et al*. Regulatory T cell frequency and modulation of IFN-gamma and IL-17 in active and latent tuberculosis. *Tuberculosis (Edinb)*. **90**, 252-61 (2010).

89. Hougardy, J. *et al*. Regulatory T cells depress immune responses to protective antigens in active tuberculosis. *Am. J. Respir. Crit. Care. Med*. **176**, 409-16 (2007).

90. Guyot-Revol, V., Innes, J.A., Hackforth, S., Hinks, T. & Lalvani, A. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *Am. J. Respir. Crit. Care. Med*. **173**, 803-10 (2006).

91. Kursar, M. *et al*. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. *J. Immunol*. **178**, 2661-2665 (2007).

92. Shafiani, S., Tucker-Heard, G., Kariyone, A., Takatsu, K. & Urdahl, K.B. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J. Exp. Med*. **207**, 1409-20 (2010).

93. Wang, J., Huizinga, T.W.J. & Toes, R.E.M. De novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid. *J. Immunol*. **183**, 4119-26 (2009).

94. Fantini, M.C. *et al*. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J. Immunol*. **172**, 5149-53 (2004).

95. Coombes, J.L. *et al*. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J. Exp. Med*. **204**, 1757-64 (2007).

96. Sun, C. *et al*. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J. Exp. Med*. **204**, 1775-85 (2007).

97. Agrawal, S. *et al*. Cutting edge: different toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol*. **171**, 4984-4989 (2003).

98. Pulendran, B., Tang, H. & Manicassamy, S. Programming dendritic cells to induce Th2 and tolerogenic responses. *Nat. Immunol*. **11**, 647-55 (2010).

99. Puig-Kröger, A. *et al*. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood* **98**, 2175-2182 (2001).

100. Hayashi, F., Yanagawa, Y., Onoé, K. & Iwabuchi, K. Dendritic cell differentiation with prostaglandin E results in selective attenuation of the extracellular signal-related kinase pathway and decreased interleukin-23 production. *Immunology* **131**, 67-76 (2010).

101. Gringhuis, S.I. *et al*. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB. *Immunity* **26**, 605-16 (2007).

102. Yi, A. *et al*. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. *J. Immunol*. **168**, 4711-20 (2002).

103. Dillon, S. *et al*. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J. Immunol*. **172**, 4733-43 (2004).

104. Pathak, S.K. *et al*. Mycobacterium tuberculosis lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. *J. Biol. Chem*. **280**, 42794-800 (2005).

105. Kobayashi, K. *et al*. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* **110**, 191-202 (2002).

106. Yang, C. *et al*. Differential regulation of interleukin-12 and tumour necrosis factor-alpha by phosphatidylinositol 3-kinase and ERK 1/2 pathways during Mycobacterium tuberculosis infection. *Clin. Exp. Immunol*. **143**, 150-60 (2006).

107. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med*. **179**, 1109-1118 (1994).

108. Schreibelt, G. *et al*. Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. *Cancer. Immunol. Immunother*. **59**, 1573-82 (2010).

109. Wang, J.E. *et al*. An 11-color flow cytometric assay for identifying, phenotyping, and assessing endocytic ability of peripheral blood dendritic cell subsets in a single platform. *J. Immunol. Methods* **341**, 106-16 (2009).

110. Autissier, P., Soulas, C., Burdo, T.H. & Williams, K.C. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. *Cytometry A* **77**, 410-9 (2010).

111. Demedts, I.K., Brusselle, G.G., Vermaelen, K.Y. & Pauwels, R.A. Identification and characterization of human pulmonary dendritic cells. *Am. J. Respir. Cell. Mol. Biol*. **32**, 177-184 (2005).

112. Masten, B.J. *et al*. Characterization of myeloid and plasmacytoid dendritic cells in human lung. *J. Immunol*. **177**, 7784-93 (2006).

113. Ten Berge, B. *et al*. A novel method for isolating dendritic cells from human bronchoalveolar lavage fluid. *J. Immunol. Methods* **351**, 13-23 (2009).

114. Bratke, K. *et al*. Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge. *Thorax* **62**, 168-175 (2007).

115. Varol, C. *et al*. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J. Exp. Med*. **204**, 171-180 (2007).

116. McMichael, A.J., Borrow, P., Tomaras, G.D., Goonetilleke, N. & Haynes, B.F. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat. Rev. Immunol*. **10**, 11-23 (2009).

117. Deng, H. *et al*. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-6 (1996).

118. Feng, Y., Broder, C.C., Kennedy, P.E. & Berger, E.A. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-7 (1996).

119. Dalgleish, A.G. *et al*. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**, 763-7

120. Wyatt, R. *et al*. The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705-11 (1998).

121. Melikyan, G.B. *et al*. Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J. Cell. Biol.* **151**, 413-23 (2000).

122. Markosyan, R.M., Cohen, F.S. & Melikyan, G.B. HIV-1 envelope proteins complete their folding into six-helix bundles immediately after fusion pore formation. *Mol. Biol. Cell.* **14**, 926-38 (2003).

123. Rambaut, A., Posada, D., Crandall, K.A. & Holmes, E.C. The causes and consequences of HIV evolution. *Nat. Rev. Genet.* **5**, 52-61 (2004).

124. Rieder, P. *et al*. Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) Diversity and Tropism in 145 Patients With Primary HIV-1 Infection. *Clin. Infect. Dis.* **1**, 1271-1279 (2011).

125. van't Wout, A.B. *et al*. Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. *J. Clin. Invest.* **94**, 2060-2067 (1994).

126. Zaitseva, M.B. *et al*. CXCR4 and CCR5 on human thymocytes: biological function and role in HIV-1 infection. *J. Immunol.* **161**, 3103-13 (1998).

127. Blaak, H. *et al*. In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline. *Proc. Natl. Acad. Sci. USA* **97**, 1269-74 (2000).

128. Salazar-Gonzalez, J.F. *et al*. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J. Exp. Med.* **206**, 1273-89 (2009).

129. Bol, S.M., Cobos-Jiménez, V., Kootstra, N.A. & van 't Wout, A.B. HIV-1 and the macrophage. *Future Virol.* **6**, 187-208 (2011).

130. Brenchley, J.M. *et al*. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J. Exp. Med.* **200**, 749-59 (2004).

131. Veazey, R.S. *et al*. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280**, 427-31 (1998).

132. Mattapallil, J.J. *et al*. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* **434**, 1093-7 (2005).

133. Swingler, S., Mann, A.M., Zhou, J., Swingler, C. & Stevenson, M. Apoptotic killing of HIV-1-infected macrophages is subverted by the viral envelope glycoprotein. *PLoS Pathog.* **3**, 1281-90 (2007).

134. Olivetta, E. & Federico, M. HIV-1 Nef protects human-monocyte-derived macrophages from HIV-1-induced apoptosis. *Exp. Cell. Res.* **312**, 890-900 (2006).

135. Carr, J.M., Hocking, H., Li, P. & Burrell, C.J. Rapid and efficient cell-to-cell transmission of human immunodeficiency virus infection from monocyte-derived macrophages to peripheral blood lymphocytes. *Virology* **265**, 319-29 (1999).

136. Mann, D.L., Gartner, S., Le Sane, F., Buchow, H. & Popovic, M. HIV-1 transmission and function of virus-infected monocytes/macrophages. *J. Immunol.* **144**, 2152-8 (1990).

137. Jameson, B. *et al.* Expression of DC-SIGN by Dendritic Cells of Intestinal and Genital Mucosae in Humans and Rhesus Macaques Expression of DC-SIGN by Dendritic Cells of Intestinal and Genital Mucosae in Humans and Rhesus Macaques. *J. Virol.* **76**, 1866-1875 (2002).

138. Li, Q. *et al.* Glycerol monolaurate prevents mucosal SIV transmission. *Nature* **458**, 1034-1038 (2009).

139. Granelli-Piperno, A., Golebiowska, A., Trumpfheller, C., Siegal, F.P. & Steinman, R.M. HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *Proc. Natl. Acad. Sci. USA* **101**, 7669-7674 (2004).

140. Fonteneau, J.F. *et al.* Human immunodeficiency virus type 1 activates plasmacytoid dendritic cells and concomitantly induces the bystander maturation of myeloid dendritic cells. *J. Virol.* **78**, 5223-5232 (2004).

141. Beignon, A.S. *et al.* Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor – viral RNA interactions. *J. Clin. Invest.* **115**, 1-11 (2005).

142. Meier, A. *et al.* Upregulation of PD-L1 on monocytes and dendritic cells by HIV-1 derived TLR ligands. *AIDS* **22**, 655-8 (2008).

143. Xu, H. *et al.* Increased B7-H1 expression on dendritic cells correlates with programmed death 1 expression on T cells in simian immunodeficiency virus-infected macaques and may contribute to T cell dysfunction and disease progression. *J. Immunol.* **185**, 7340-8 (2010).

144. Swiecki, M. & Colonna, M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol. Rev.* **234**, 142-162 (2010).

145. Karlin, S., Doerfler, W. & Cardon, L.R. Why is CpG suppressed in the genomes of virtually all small eukaryotic viruses but not in those of large eukaryotic viruses? *J. Virol.* **68**, 2889-2897 (1994).

146. Berg, R.K. *et al.* Genomic HIV RNA induces innate immune responses through RIG-I-dependent sensing of secondary-structured RNA. *PloS one* **7**, e29291 (2012).

147. Martinelli, E. *et al.* HIV-1 gp120 inhibits TLR9-mediated activation and IFN-alpha secretion in plasmacytoid dendritic cells. *Proc. Natl. Acad. Sci. USA* **104**, 3396-3401 (2007).

148. Geijtenbeek, T.B.H. & Gringhuis, S.I. Signalling through C-type lectin receptors: shaping immune responses. *Nat. Rev. Immunol.* **9**, 465-479 (2009).

149. Shan, M. *et al.* HIV-1 gp120 mannoses induce immunosuppressive responses from dendritic cells. *PLoS Pathog.* **3**, e169 (2007).

150. Izaguirre, A. *et al.* Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *J. Leukoc. Biol.* **74**, 1125-1138 (2003).

151. Gerosa, F. *et al.* The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J. Immunol.* **174**, 727-34 (2005).

152. Hibbert, L., Pflanz, S., Malefyt, R.W. & Kastelein, R.A. IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells. *J. Interferon Cytokine Res.* **23**, 513-522 (2003).

153. Gurney, K.B., Colantonio, A.D., Blom, B., Spits, H. & Uittenbogaart, C.H. Endogenous IFN-alpha production by plasmacytoid dendritic cells exerts an antiviral effect on thymic HIV-1 infection. *J. Immunol.* **173**, 7269-7276 (2004).

154. Meyers, J.H. *et al.* Impact of HIV on cell survival and antiviral activity of plasmacytoid dendritic cells. *PloS one* **2**, e458 (2007).

155. Stary, G. *et al.* Plasmacytoid dendritic cells express TRAIL and induce CD4+ T-cell apoptosis in HIV-1 viremic patients. *Blood* **114**, 3854-3863 (2009).

156. Herbeuval, J.P. *et al.* Differential expression of IFN-alpha and TRAIL/DR5 in lymphoid tissue of progressor versus nonprogressor HIV-1-infected patients. *Proc. Natl. Acad. Sci. USA* **103**, 7000-7005 (2006).

157. Manches, O. *et al.* HIV-activated human plasmacytoid DCs induce Tregs through an indoleamine 2,3-dioxygenase-dependent mechanism. *J. Clin. Invest.* **118**, 3431-9 (2008).

158. Boasso, A. *et al.* HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells. *Blood* **109**, 3351-9 (2007).

159. Raska, M. *et al.* Glycosylation patterns of HIV-1 gp120 depend on the type of expressing cells and affect antibody recognition. *J. Biol. Chem.* **285**, 20860-20869 (2010).

160. Torrado, E. & Cooper, A.M. IL-17 and Th17 cells in tuberculosis. *Cytokine Growth F. R.* **21**, 455-462 (2010).

161. Redford, P.S., Murray, P.J. & O'Garra, A. The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunol.* **4**, 261-70 (2011).

162. Iwasaki, A. & Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *Science* **327**, 291-5 (2010).

163. Underhill, D., Ozinsky, A., Smith, K. & Aderem, A. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA* **96**, 14459-14463 (1999).

164. Ishikawa, H., Ma, Z. & Barber, G.N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788-92 (2009).

165. Gautier, G. *et al.* A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J. Exp. Med.* **201**, 1435-46 (2005).

166. Doz, E. *et al.* Mycobacterial phosphatidylinositol mannosides negatively regulate host Toll-like receptor 4, MyD88-dependent proinflammatory cytokines, and TRIF-dependent co-stimulatory molecule expression. *J. Biol. Chem.* **284**, 23187-96 (2009).

167. Driessen, N.N. *et al.* Role of phosphatidylinositol mannosides in the interaction between mycobacteria and DC-SIGN. *Infect. Immun.* **77**, 4538-47 (2009).

168. Gregori, S. *et al.* Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* **116**, 935-944 (2010).

169. Steinbrink, K., Wölfl, M., Jonuleit, H., Knop, J. & Enk, A.H. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* **159**, 4772-4780 (1997).

170. Bartz, H., Avalos, N.M., Baetz, A., Heeg, K. & Dalpke, A.H. Involvement of suppressors of cytokine signaling in toll-like receptor-mediated block of dendritic cell differentiation. *Blood* **108**, 4102-4108 (2006).

171. Balboa, L. *et al*. Mycobacterium tuberculosis impairs dendritic cell response by altering CD1b, DC-SIGN and MR profile. *Immunol. Cell. Biol.* **88**, 716-726 (2010).

172. Lauener, R.P., Goyert, S.M., Geha, R.S. & Vercelli, D. Interleukin 4 down-regulates the expression of CD14 in normal human monocytes. *Eur. J. Immunol.* **20**, 2375-81 (1990).

173. Martin, B., Hirota, K., Cua, D.J., Stockinger, B. & Veldhoen, M. Interleukin-17-producing gamma delta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* **31**, 321-30 (2009).

174. van Haarst, J. *et al*. CD1a+ and CD1a- accessory cells from human bronchoalveolar lavage differ in allostimulatory potential and cytokine production. *Am. J. Respir. Cell. Mol. Biol.* **15**, 752-759 (1996).

175. van Haarst, J.M., de Wit, H.J., Drexhage, H.A. & Hoogsteden, H.C. Distribution and immunophenotype of mononuclear phagocytes and dendritic cells in the human lung. *Am. J. Respir. Cell. Mol. Biol.* **10**, 487-492 (1994).

176. Cheong, C. *et al*. Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas. *Cell* **143**, 416-29 (2010).

177. Schleifer, K.H. & Kandler, O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**, 407-77 (1972).

178. Inohara, N. *et al*. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* **278**, 5509-12 (2003).

179. Chamaillard, M. *et al*. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat. Immunol.* **4**, 702-7 (2003).

180. Schnurr, M. *et al*. Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway. *Blood* **105**, 1582-9 (2005).

181. Kolb-Mäaurer, A. *et al*. Production of IL-12 and IL-18 in human dendritic cells upon infection by Listeria monocytogenes. *FEMS Immunol. Med. Microbiol.* **35**, 255-262 (2003).

182. Bazil, V. & Strominger, J.L. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *J. Immunol.* **147**, 1567-1574 (1991).

183. Bazil, V. Physiological enzymatic cleavage of leukocyte membrane molecules. *Immunol. Today* **16**, 135-40 (1995).

184. Zanoni, I. *et al*. CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation. *Nature* **460**, 264-8 (2009).

185. Fujii, S., Liu, K., Smith, C., Bonito, A.J. & Steinman, R.M. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J. Exp. Med.* **199**, 1607-1618 (2004).

186. Flynn, J.L. & Chan, J. Immunology of tuberculosis. *Annu. Rev. Immunol.* **19**, 93-129 (2001).

187. Dieu, M.C. *et al.* Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* **188**, 373-386 (1998).

188. Provoost, S., Maes, T., Joos, G.F. & Tournoy, K.G. Monocyte-derived dendritic cell recruitment and allergic T(H)2 responses after exposure to diesel particles are CCR2 dependent. *J. Allergy Clin. Immun.* **129**, 483-91 (2011).

189. Robays, L.J. *et al.* Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation. *J. Immunol.* **178**, 5305-5311 (2007).

190. Wölfle, S.J. *et al.* PD-L1 expression on tolerogenic APCs is controlled by STAT-3. *Eur. J. Immunol.* **41**, 413-424 (2011).

191. Bandukwala, H.S. *et al.* Signaling through Fc gamma RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. *J. Exp. Med.* **204**, 1875-1889 (2007).

192. Ilarregui, J.M. *et al.* Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat. Immunol.* **10**, 981-991 (2009).

193. Geissmann, F. *et al.* Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J. Exp. Med.* **187**, 961-966 (1998).

194. Gogolak, P. *et al.* Differentiation of CD1a- and CD1a+ monocyte-derived dendritic cells is biased by lipid environment and PPARgamma. *Blood* **109**, 643-652 (2007).

195. Cernadas, M., Lu, J., Watts, G. & Brenner, M.B. CD1a expression defines an interleukin-12 producing population of human dendritic cells. *Clin. Exp. Immunol.* **155**, 523-533 (2009).

196. Kalinski, P., Wieckowski, E., Muthuswamy, R. & de Jong, E. Generation of stable Th1/CTL-, Th2-, and Th17-inducing human dendritic cells. *Methods. Mol. Biol.* **595**, 117-133 (2010).

197. Kaufmann, S.H.E., Hussey, G. & Lambert, P. New vaccines for tuberculosis. *Lancet* **375**, 2110-2119 (2010).

198. Sieling, P.A. *et al.* IL-12 Regulates T Helper Type 1 Cytokine Responses in Human Infectious Disease. *J. Immunol.* **153**, 3639-3947 (1994).

199. Ridley, D.S. & Jopling, W.H. Classification of leprosy according to immunity. A five-group system. *Int. J. Leprosy* **34**, 255-273 (1966).

200. Ueno, H. *et al.* Harnessing human dendritic cell subsets for medicine. *Immunol. Rev.* **234**, 199-212 (2010).

201. Chang, C.C., Wright, A. & Punnonen, J. Monocyte-derived CD1a+ and CD1a- dendritic cell subsets differ in their cytokine production profiles, susceptibilities to transfection, and capacities to direct Th cell differentiation. *J. Immunol.* **165**, 3584-3591 (2000).

202. Kvistborg, P. *et al.* Comparison of monocyte-derived dendritic cells from colorectal cancer patients, non-small-cell-lung-cancer patients and healthy donors. *Vaccine* **28**, 542-547 (2009).

203. da Costa, T.B., Sardinha, L.R., Larocca, R., Peron, J.P.S. & Rizzo, L.V. Allogeneic apoptotic thymocyte-stimulated dendritic cells expand functional regulatory T cells. *Immunology* **133**, 123-132 (2011).

204. Chen, X., Doffek, K., Sugg, S.L. & Shilyansky, J. Phosphatidylserine regulates the maturation of human dendritic cells. *J. Immunol.* **173**, 2985-2994 (2004).

205. Rajashree, P., Supriya, P. & Das, S.D. Differential migration of human monocyte-derived dendritic cells after infection with prevalent clinical strains of Mycobacterium tuberculosis. *Immunobiology* **213**, 567-75 (2008).

206. Scandella, E., Men, Y., Gillessen, S., Förster, R. & Groettrup, M. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* **100**, 1354-61 (2002).

207. Boersema, P.J., Raijmakers, R., Lemeer, S., Mohammed, S. & Heck, A.J.R. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat. protoc.* **4**, 484-94 (2009).

208. Søndergaard, J.N. *et al*. Differential sensitivity of melanoma cell lines with BRAFV600E mutation to the specific Raf inhibitor PLX4032. *J. Transl. Med.* **8**, 39 (2010).

209. Zhu, X., Borchers, C., Bienstock, R.J. & Tomer, K.B. Mass spectrometric characterization of the glycosylation pattern of HIV-gp120 expressed in CHO cells. *Biochemistry* **39**, 11194-204 (2000).

210. Wei, X. *et al*. Antibody neutralization and escape by HIV-1. *Nature* **422**, 307-12 (2003).

211. Forsell, M.N.E., Schief, W.R. & Wyatt, R.T. Immunogenicity of HIV-1 envelope glycoprotein oligomers. *Curr. Opin. HIV AIDS* **4**, 380-7 (2009).

212. Doores, K.J. *et al*. Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens. *Proc. Natl. Acad. Sci. USA* **107**, 13800-13805 (2010).

213. Turville, S.G. *et al*. HIV gp120 receptors on human dendritic cells. *Blood* **98**, 2482-2488 (2001).

214. Turville, S.G. *et al*. Diversity of receptors binding HIV on dendritic cell subsets. *Nat. Immunol.* **3**, 975-83 (2002).

215. Altfeld, M., Fadda, L., Frleta, D. & Bhardwaj, N. DCs and NK cells: critical effectors in the immune response to HIV-1. *Nat. Rev. Immunol.* **11**, 176-186 (2011).

216. Lambert, A.A., Gilbert, C., Richard, M., Beaulieu, A.D. & Tremblay, M.J. The C-type lectin surface receptor DCIR acts as a new attachment factor for HIV-1 in dendritic cells and contributes to trans- and cis-infection pathways. *Blood* **112**, 1299-307 (2008).

217. Bess, J.W., Gorelick, R.J., Bosche, W.J., Henderson, L.E. & Arthur, L.O. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. *Virology* **230**, 134-44 (1997).

218. Nielsen, C.M., Bygbjerg, I.C. & Vestergaard, B.F. Detection of HIV antigens in eluates from whole blood collected on filterpaper. *Lancet* **1**, 566-567 (1987).

219. Drosten, C. *et al*. Ultrasensitive monitoring of HIV-1 viral load by a low-cost real-time reverse transcription-PCR assay with internal control for the 5' long terminal repeat domain. *Clin. Chem.* **52**, 1258-1266 (2006).

220. Edelmann, A. *et al*. Improvement of an ultrasensitive human immunodeficiency virus type 1 real-time reverse transcriptase-polymerase chain reaction targeting the long terminal repeat region. *Transfusion* **50**, 685-692 (2010).

221. Blackburn, S.D. *et al*. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat. Immunol*. **10**, 29-37 (2009).

222. Pancera, M. *et al*. Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. *Proc. Natl. Acad. Sci. USA* **107**, 1166-1171 (2010).

223. Patterson, S., Rae, A., Hockey, N., Gilmour, J. & Gotch, F. Plasmacytoid dendritic cells are highly susceptible to human immunodeficiency virus type 1 infection and release infectious virus. *J. Virol*. **75**, 6710-6713 (2001).

224. O'Brien, M. *et al*. Spatiotemporal trafficking of HIV in human plasmacytoid dendritic cells defines a persistently IFN-alpha–producing and partially matured phenotype. *J. Clin. Invest*. **121**, 1088-1101 (2011).

225. Smed-Sörensen, A. *et al*. Differential Susceptibility to Human Immunodeficiency Virus Type 1 Infection of Myeloid and Plasmacytoid Dendritic Cells. *J. Virol*. **79**, 8861-8869 (2005).

226. Pacanowski, J. *et al*. Reduced blood CD123+ (lymphoid) and CD11c+ (myeloid) dendritic cell numbers in primary HIV-1 infection. *Blood* **98**, 3016-3021 (2001).

227. Soumelis, V. *et al*. Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients. *Blood* **98**, 906-912 (2001).

228. Chehimi, J. *et al*. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. *J. Immunol*. **168**, 4796-4801 (2002).

229. Brown, K.N., Wijewardana, V., Liu, X. & Barratt-Boyes, S.M. Rapid influx and death of plasmacytoid dendritic cells in lymph nodes mediate depletion in acute simian immunodeficiency virus infection. *PLoS Pathog*. **5**, e1000413 (2009).

230. Malleret, B. *et al*. Primary infection with simian immunodeficiency virus: plasmacytoid dendritic cell homing to lymph nodes, type I interferon, and immune suppression. *Blood* **112**, 4598-4608 (2008).

231. Nascimbeni, M. *et al*. Plasmacytoid dendritic cells accumulate in spleens from chronically HIV-infected patients but barely participate in interferon-alpha expression. *Blood* **113**, 6112-6119 (2009).

232. Mortellaro, A., Robinson, L. & Ricciardi-Castagnoli, P. Spotlight on Mycobacteria and dendritic cells: will novel targets to fight tuberculosis emerge? *EMBO Mol. Med*. **1**, 19-29 (2009).

233. van de Vosse, E., Hoeve, M.A. & Ottenhoff, T.H.M. Human genetics of intracellular infectious diseases: molecular and cellular immunity against mycobacteria and salmonellae. *Lancet Infect. Dis*. **4**, 739-749 (2004).

234. Jung, Y., Ryan, L., LaCourse, R. & North, R.J. Differences in the ability to generate type 1 T helper cells need not determine differences in the ability to resist Mycobacterium tuberculosis infection among mouse strains. *J. Infect. Dis*. **199**, 1790-6 (2009).

235. Lowe, D.M., Redford, P.S., Wilkinson, R.J., O'Garra, A. & Martineau, A.R. Neutrophils in tuberculosis: friend or foe? *Trends Immunol*. **33**, 14-25 (2012).

236. Lai, X. *et al*. Immune biology of macaque lymphocyte populations during mycobacterial infection. *Clin. Exp. Immunol*. **133**, 182-92 (2003).

237. Moog, C. *et al*. Neutralization of primary human immunodeficiency virus type 1 isolates: a study of parameters implicated in neutralization in vitro. *AIDS Res. Hum. Retrov*. **13**, 19-27 (1997).

238. Schuurhuis, D.H. *et al*. In situ expression of tumor antigens by messenger RNA-electroporated dendritic cells in lymph nodes of melanoma patients. *Cancer Res*. **69**, 2927-34 (2009).