# Investigating the role of the pulmonary innate immune system in anti-tuberculosis immunity

# Investigating the role of the pulmonary innate immune system in anti-tuberculosis immunity

By Rocky Lai, B.Sc

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree Doctor of Philosophy

**McMaster University**

**DOCTOR OF PHILOSOPHY (2017) McMaster University**

**Medical Sciences – Infection and Immunity. Hamilton, Ontario, Canada**

**TITLE:** Investigating the role of the pulmonary innate immune system in anti-tuberculosis immunity

**AUTHOR:** Rocky Lai B.Sc. (McMaster University)

**SUPERVISOR:** Dr. Zhou Xing

**NUMBER OF PAGES:** 162 (including title)

## Abstract

*M.tb*, the causative agent of pulmonary tuberculosis (TB) remains one of the leading causes of infectious disease-based death worldwide. BCG, the only clinically approved TB vaccine, has been in use for almost a century to vaccinate against TB. Despite its success in protecting against disseminated forms of TB, it is unable to provide protection against pulmonary *M.tb* infection. Although there have been many recent efforts to enhance or replace BCG, our lack of understanding towards host immunity against *M.tb* has substantially hindered this goal. One aspect of pulmonary *M.tb* infection that remains poorly understood is the induction of Th1 immunity, which is substantially delayed in comparison to other pulmonary infections. This allows the bacteria to establish an infectious foothold within the host and impairs the ability of the host to clear the infection. Given the importance of the innate immune response in the induction of adaptive immunity, this delay in the establishment of Th1 immunity following pulmonary *M.tb* infection is likely due to a defect in the early innate immune response. However, the specific roles of this immune compartment in regards to T cell activation following pulmonary *M.tb* infection is still not well understood. As such, the scope of this thesis is to gain an increased understanding towards the role of the innate immune compartment in the generation of Th1 responses. Such insights will allow us to develop new strategies to improve upon future and existing TB vaccine design.

## Acknowledgements

I would like to express my thanks to everyone who has helped me in the past and present, both within the lab as well as MIRC. In particular:

Dr. Mangalakumari Jeyanathan, for all the scientific mentorship and advice over the years.

Anna Zganiacz, for keeping the lab (and level 3) running, so that I didn't have to.

The rest of the Xing lab, past and present, but especially Sam Afkhami, Amandeep Khera, Dr. Yushi Yao, Maryam Vaseghi-Shanjani, Dr. Ryan Shaler and Dr. Daniela Damjanovic, for being there for scientific interactions and helping with any random thing.

All past and present trainees, faculty and technical staff within MIRC, for whenever I need to borrow reagents, help with troubleshooting, or just vent.

Hong Liang, for training me on the Influx in level 3, and for keeping the flow cytometers running.

My committee members, Dr. Martin Stampfli and Dr. Yonghong Wan, for their mentorship and advice.

My parents, for understanding that a Ph.D takes a long time to complete, and…

Finally, my supervisor, Dr. Zhou Xing, for his continuing support, guidance, and for giving me a chance all those years ago.

## List of figures

## List of abbreviations

(c)RPMI media – (complete) Roswell Park Memorial Institute medium

AdHu5 – human adenovirus serotype 5

Ag – antigen

Ag85A – antigen 85A

AM – alveolar macrophage

ANOVA – analysis of variance

APC – antigen presenting cell

BAL – bronchioalveolar lavage

Batf - basic leucine zipper transcription factor ATF-like 3

BCG – Bacille Calmette-Guérin

BST2 - bone marrow stromal antigen 2

CD – cluster of differentiation

CDP – common DC progenitor

cDC – conventional DC

CFU – colony forming unit

CMP – common myeloid progenitor

DC – dendritic cell

dLN – draining lymph node

FBS – fetal bovine serum

FimH – fimbrael adhesin H

FLT3 – Fms-like tyrosine kinase 3

HDM – house dust mite

HIV – Human Immunodeficiency Virus

i.m. – intramuscular

i.n. – intranasal

i.p. – intraperitoneal

i.t. – intratracheal

IFN – interferon

Kbp – kilobase pairs

Ig – immunoglobulin

IL– interleukin

IM – interstitial macrophage

IP-10 – interferon gamma-induced protein 10

IRF – interferon regulatory factor

JAK – janus-kinase

KC – keratinocyte-derived chemokine

*M.tb – Mycobacterium tuberculosis*

manLAM – mannose-capped lipoarabinomannan

MCP-1 – monocyte chemotactic protein 1

MDR – multi-drug resistant

MDP – macrophage and DC progenitor

MHC-II – major Histocompatibility Complex II

MIP – macrophage inflammatory protein

moDC – monocyte derived DC

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells -

NOD - nucleotide-binding oligomerization domain

NTM – non-tuberculous mycobacteria

PBS – phosphate-buffered saline

pDC – plasmacytoid DC

PRR – Pattern recognition receptor

rDC – lymphoid resident DC

RSV - respiratory Syncytial Virus

SCID - severe combined immunodeficiency

SD – standard deviation

SEM – standard error of mean

Siglec – sialic acid-binding immunoglobulin-like lectin

SLO – secondary lymphoid organ

STAT – signal transducer and activator of transcription

TB – tuberculosis

Th1 – T helper 1

TLR- toll-like receptor

TNF – tumour Necrosis Factor

Treg – regulatory T cell

VLA-A – very late antigen 1

VSV – vesicular stomatitis virus

XDR – extensively drug resistant

## Declaration of academic achievements

**Chapter 3**: Restoration of innate immune activation accelerates Th1 priming and protection following pulmonary mycobacterial infection

**Rocky Lai**, Mangalakumari Jeyanathan, Christopher R. Shaler, Daniela Damjanovic, Amandeep Khera, Carly Horvath, Ali A. Ashkar and Zhou Xing. Eur. J. Immunol, 44(5):1375-86.

R.L. M.J. and Z.X. conceived and designed the study. R.L. performed most of the experiments with help from C.S., D.D., A.K. and C.H. R.L performed data analysis. A.A. provided critical reagents. R.L. and Z.X wrote the manuscript

**Chapter 4**: CD11b+ dendritic cell-mediated anti-*M.tuberculosis* Th1 activation is counter-regulated by CD103+ dendritic cells via interleukin-10

**Rocky Lai,** Manglakumairi Jeyanathan, Sam Afkhami, Anna Zganiacz, Joanne A Hammill, Yushi Yao, Charu Kaushic, Zhou Xing

R.L., M.J. and Z.X. conceived and designed the study. R.L. performed most of the experiments, with help from S.A., A.Z., J.A.H. and Y.Y. R.L. and M.J. performed data analysis. C.K. provided critical reagents. R.L., M.J. and Z.X. wrote the manuscript.

**Chapter 5**: Mucosal imprinting of host innate immunity following AdHuAg85A vaccination protects against pulmonary *M.tb* infection independently of T cell responses.

**Rocky Lai**, Sam Afkhami, Manglakumari Jeyanathan, Anna Zganiacz, Zhou Xing

R.L and M.M. conceived and designed the study. R.L. performed most of the experiments, with help from S.A. and A.Z. R.L. and Z.X. wrote the manuscript.

# Table of Contents

# Chapter 1. Introduction

## 1.1. General introduction

*Mycobacterium tuberculosis* (*M.tb*) is the causative agent of pulmonary tuberculosis (TB) and remains the leading cause of death due to infectious disease worldwide. Since its initial discovery by Robert Koch in 1882, our understanding towards the control of the disease has increased dramatically, to the point where the majority of TB cases have the potential to be successfully treated. Despite these advances, approximately one third of the world's population remains latently infected with the *M.tb*, with 5-10% of infected individuals eventually developing clinically active disease. In 2016 there were an estimated 10.4 million new cases and 1.8 million deaths recorded, highlighting the fact that new cases continue to emerge despite our ability to treat the infection. A large portion of the afflicted population are situated in sub-Saharan Africa and Southeast Asia, although due to increased globalization this is no longer merely a regional issue (*1*). As of the year 2016, TB ranks above HIV as the leading cause of global deaths due to an infectious disease (*1*).

Despite the enormity of the global TB burden, 90-95% of exposed individuals will adequately control the bacteria and not develop clinical disease. A subset of individuals exposed to TB (~20-25%) demonstrate early clearance of *M.tb* and eliminate the pathogen before it is able to establish an infection (*2*). The remaining 75-80% of individuals will be able to control but not eliminate the infection and develop latent TB (*1*). About 5-10% of infected individuals will experience reactivation within their lifetime, usually due to a loss of control in TB when an individual becomes immune compromised such as during HIV infection or when given anti-TNF therapy (*1*, *3*).

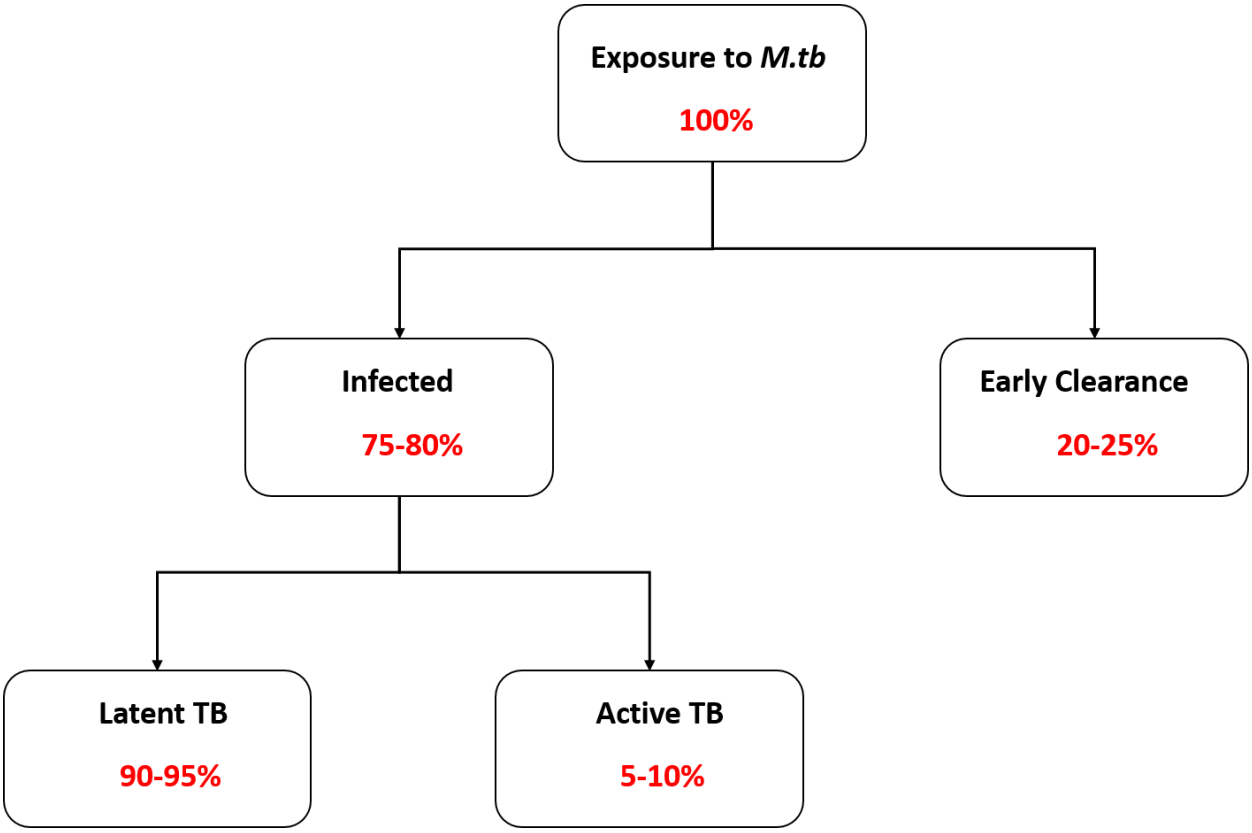



**Figure 1 – Possible outcomes following *M.tb* exposure**. Most individuals exposed to *M.tb* will adequately control the infection throughout their lifetime, resulting in latent TB and has a minimum impact on their daily health. A loss of containment will lead to the development of active TB.

1.1.1. Current treatment options for TB

Currently, the standard treatment option available for drug-susceptible TB infection is composed of a 6-9 month antibiotic regimen of rifampicin and isoniazid. (*4*). However, the recent emergence of multi-drug resistance (MDR) and extensively-drug resistant (XDR) forms of TB has made it increasingly difficult to treat *M.tb* infection. Although additional antibiotic regiments are available, vaccines have recently become an increasingly attractive alternative method to control *M.tb*, and indeed the use of Bacille Calmette-Guérin (BCG) over the last 80 years has led to an substantial decrease in *M.tb* burden, particularly in protection against disseminated childhood forms of TB.

BCG was developed by Albert Calmette and his associate Camille Guérin at the Pasteur Institute de Lille when they noticed that sub-culturing of tubercule bacilli lead to a reduction in virulence (*5*). Starting with a virulent bovine strain of TB (*M. bovis*), they proceeded to sub-culture the bacteria until finally by 1921 they had generated a strain that was unable to produce active TB when injected into various animal models. Although the protocol and strains of BCG used varies between countries, it is usually given once subcutaneously shortly after birth (within 12 months) at a dose of $4x10^5$ colony forming units per host. However, as it is a live-attenuated bacterium, WHO guidelines dictate that children who may be HIV-positive do not receive BCG (*6*). While there were some initial setbacks, by the 1950s BCG was used routinely in most major countries around the world. Currently, with the exception of a few countries such as Canada and the United States, it is used in more than 180 countries and over 3 billion children are immunized around the world (*5*, *7*) .

However, despite the widespread use and success of BCG in protection against childhood forms of disseminated TB, BCG has repeatedly been shown to have poor protective efficacy

towards adult pulmonary TB (*8*). The failure of BCG to protect against pulmonary TB, along with the recent emergence of antibiotic resistant TB, underscores the need for the development of new vaccines and vaccination strategies that will be able to provide adequate protection against *M.tb*. Although many vaccines are currently in the developmental pipeline (*9*), our lack of mechanistic insights towards the generation of protective immunity against *M.tb* has severely impeded our progress towards this goal.

1.1.2. Pathogenesis of tuberculosis infection

*M.tb* is a facultative intracellular bacterium that spreads through aerosols containing viable *M.tb* bacilli generated by patients displaying active TB, whereby it is inhaled and deposited within the lung alveoli. This is in strict contrast with latently infected individuals who display no apparent symptoms and are non-contagious (*10*). A characteristic of TB is the formations of granulomas within the lung and other infected organs. These granuloma structures are considered a hallmark of TB pathology and have been identified long before the discovery of *M.tb* itself (*11*). However, it was not until 1884 after the identification of *M.tb* that the bacilli was discovered within the granuloma as well, linking the lesion with *M.tb* itself (reviewed in (*12*)). Initially composed primarily of infected macrophages, the $CD4^+$ and $CD8^+$ T cells that eventually arrive within 3 weeks post infection surrounds the core of infected macrophage (*11, 12*) and forms the perimeter of the granuloma. The secretion of activation factors such as IFN-γ by antigen-specific T cells results in a morphological change in the infected macrophage towards both an epithelial-like morphology (termed epithelioid macrophage) and giant multinucleated cells, which are formed from the fusion of infected macrophages (*12-14*). In most cases, *M.tb* is sequestered within these structures with minimal impact on host respiratory functions. However, disruption of these structures, usually when individuals become immune compromised, leads to the active TB. This is the result of disease progression, where the centres of the granulomas will

develop a necrotic focus due to the caseation of the macrophage centre (*15*). This leads to the eventual spreading of *M.tb* to other parts of the lung as well as throughout the body, further disrupting lung function as well as eventually causing much more severe forms of tuberculosis such as miliary tuberculosis or tuberculosis meningitis. These forms of TB carries with it a high morbidity and mortality, particularly among HIV-infected individuals (*16*). The loss of containment within the granuloma also results in the generation of aerosolized bacilli and subsequent transmission of *M.tb* (*17*), making these individuals highly contagious.

Although phagocytic cells are the primary targets of infection, key to protection against *M.tb* itself is the generation of adaptive T-helper 1 (Th1) lymphocytic responses (*18*). Specifically, $CD4^+$ IFN-γ T cells responses were found to be the primary mediators of anti-TB immunity (*19, 20*), and the induction of this response by BCG has been linked to its protective efficacy of the vaccine against *M.tb* (*21*). Although recent publications have put some doubt towards the importance of IFN-γ responses as a protective correlate (*22, 23*), animal models as well as clinical studies have repeatedly demonstrated the importance of IFN-γ in the control of *M.tb* infection (*24-27*). Given that $CD4^+$ T cells are critical to the production of IFN-γ following pulmonary *M.tb* infection, the priming and generation of $CD4^+$ T cell responses is critical to the generation of anti-TB responses, and eliciting this response has been a goal for anti-TB vaccine design for many years (*9*).

## 1.2. Impaired generation of Th1 responses following pulmonary *M.tb* infection

Despite the importance of Th1 immune responses in host anti-TB immunity, there is a substantial delay in the generation of such responses following infection, which are not observed at the site of infection for at least 3 weeks post infection (*18, 28*). This delay is in sharp contrast with other pulmonary infectious disease models such as influenza (*29*) and other intracellular

pathogens such as *Listeria monocytogenes* (*30*), in which the effector T cell populations are observed as early as 7-8 days post infection within the lung. The swift arrival of Th1 responses in these models correlate with a rapid control of the infection, and it is believed that this delay in Th1 induction allows *M.tb* to establish a foothold in the lung, thus preventing the host immune response from effectively controlling and eliminating *M.tb*. As such, the delayed induction of Th1 immunity has recently been recognized as another hallmark of pulmonary TB and a serious impediment towards the ability of the host to control *M.tb* infection (*18, 28, 31*). Given the importance of Th1 immunity in the control of *M.tb*, it is imperative to understand the mechanism behind the impairment of this response. Discussed below are potential checkpoints at which *M.tb* may directly or indirectly impair the generation of host Th1 responses (Summarized in **Figure 2**)

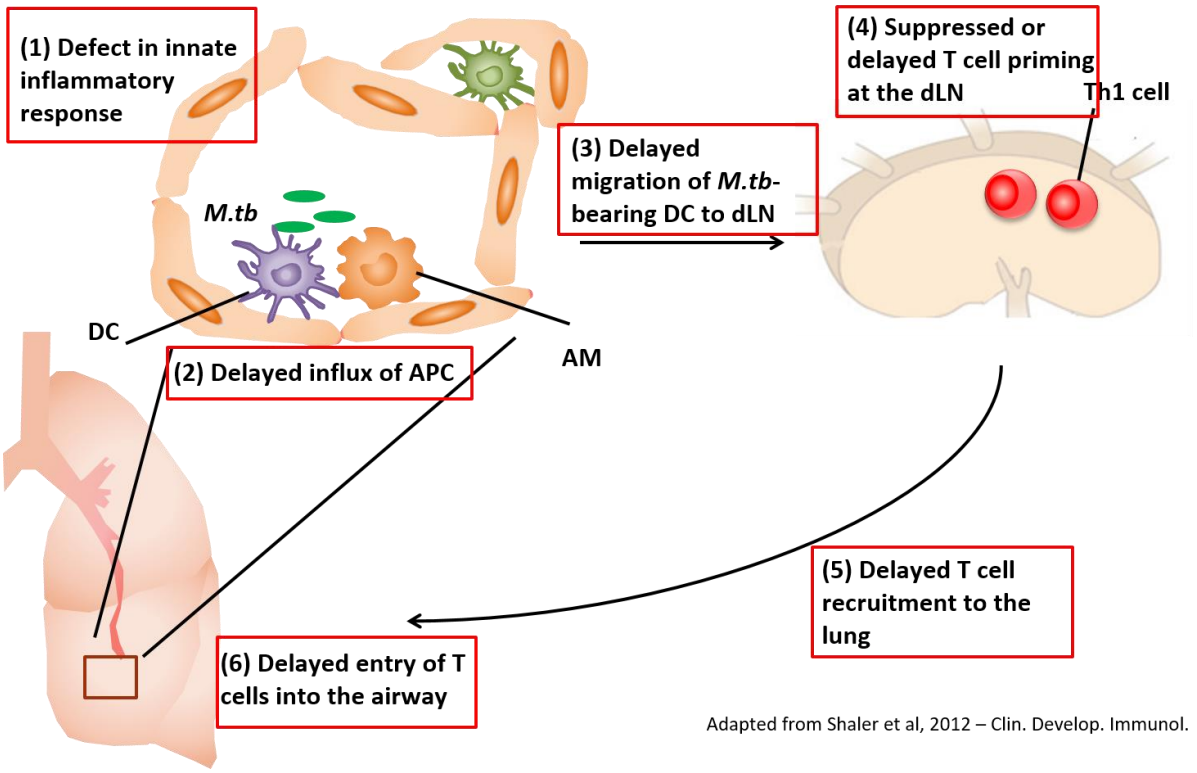



**Figure 2 – Potential checkpoints in delayed priming of Th1 responses following pulmonary *M.tb* infection**. The generation of Ag-specific Th1responses at the site of infection is dependent on many steps. A defect in any of these would result in the delayed generation of Th1 immunity. In particular, steps 1-4 represent key points that are important for Th1 priming (Reviewed in (*28*)).

## 1.3. Impaired initiation of early immune responses against *M.tb*

In the context of pulmonary *M.tb*, alveolar macrophages (AM) and interstitial macrophages (IM) are the two major macrophage populations described within the lung (*32, 33*), and are critical to both the detection and control of *M.tb*. AM are found within the alveolar airspace, and in a steady state represent 90-95% of the cell population within that space (*32, 34*). Given their anatomical location, it is believed that the main role of AM is immune surveillance of the lung, and indeed this function is reflected in their high expression of Fc and TLR receptors used for bacterial uptake and sensing (*35*). AM are particularly crucial in anti-TB immunity as these cells reside within the airway and are among the first to respond to *M.tb* upon infection (*32, 36*). AM are also unique from other macrophages in the lung due to their high expression of $CD11c^{+}$, which is traditionally associated with dendritic cells (DC). Typically, CD11c is used in conjunction with auto-fluorescence and sialic acid-binding immunoglobulin-like lectin (Siglec-F) for phenotypic identification of AM (*32*). Although AM typically have a low expression of MHC-II, upon activation the expression level of MHC-II increases significantly (*32*). Recent literature has demonstrated that AM are established shortly after birth from fetal monocytes (*37*), and are mainly replenished through self-renewal (*38, 39*). As such, they are relatively long-lived and have a turn-over rate of 40% in one year (*40*), and is contrary to the traditional view of a short lived innate phagocyte such as the monocyte which has a half life of approximately 24 hours in circulation (*39, 41*). As such, AM represent a persistent innate population at the lung that plays a major role in anti-TB immunity.

In contrast, IM are located within the parenchymal space (or interstitium) between the airways. These cells are differentiated from AM based not only from their lack of CD11c and Siglec-F, but also due to their expression of CD11b (*32*). Generally, IM are believed to be

replenished by circulating monocytes (*41, 42*), although there is also some evidence for their self-renewal capabilities (*43*). Transcriptionally, IM are very distinct from tissue-resident macrophages such as AM (*44, 45*). However, given the difficulties in the identification of IM (*45*), these cells are not as well characterized as AM in terms of their functionality apart from the "classical" functions of a macrophage such as phagocytosis. There is evidence to suggest that these cells mainly interact with DC and interstitial lymphocytes to promote immune responses (*33, 34*). Nevertheless, both macrophage populations have been demonstrated to participate in immune surveillance and early immune responses at the lung (*32, 45, 46*).

Recognition of the bacteria by these cells is mainly mediated through the use of pattern recognition receptors (PRRs) such as the toll-like receptor (TLR) family members TLR-2, 4 and 9 (*47, 48*) as well as the NOD-like receptor NOD2 (*49*) that recognize various *M.tb* components. In particular, AM express a high number of TLR molecules that serve to both recognize and permit the entry to *M.tb* into the cell (*36*). Recognition of *M.tb* by these sentinel cells initiates a signaling cascade that results in the translocation of NF-κB, ultimately leading to the maturation of the infected cell and activation of processes such as increased expression of MHC-II and co-stimulatory molecules (*50, 51*). However, there is extensive evidence demonstrating the ability of *M.tb* to inhibit the maturation process, prolonging its survival within the macrophages and preventing killing of *M.tb* (*52, 53*). This inhibition not only serves to impair the generation of *M.tb* antigens, but also inhibits the secretion of inflammatory cytokines such as TNF-α and IL-1β as well as chemokines such as MCP-1 and IP-10 (*54, 55*). This inhibition leads to the delay in key downstream immune processes, resulting in an overall delay in the initiation of the immune cascade (See **Figure 2**).

The eventual upregulation of early inflammatory response leads to a further recruitment of innate cells such as neutrophils, monocytes and DC (*28*). In particular, the recruitment of DC and other antigen presenting cells (APC) to the lung is particularly important for downstream Th1 activation. As discussed, the acquisition of antigen by these various APC populations result in the down-regulation of antigen acquisition capabilities while up-regulating molecules required for antigen presentation such as MHC-II as well as co-stimulatory molecules like CD40, CD80 and CD86 (*56*). In addition, maturation of *M.tb*-bearing DC causes them to respond to different sets of chemokines through the upregulation of CCR7 chemokine receptor while down-regulating CCR5 and CCR3 (*57*). This leads to a lost in responsiveness to chemokines such as RANTES and MIP-1α, and instead allowing them to respond to CCL19/21 secreted by the lung draining lymph node (dLN), inducing migration of these antigen-bearing cells to migrate to the dLN. Recently, Khader et al have established the importance of IL-12p40 towards CCR7 expression (*58*), highlighting the importance of this cytokine on DC migration. The same study has also demonstrated that *M.tb* itself is able to induce the secretion of IL-12p40, suggesting that the bacteria may also be using this strategy to promote dissemination towards other parts of the body as DC typically have very poor bactericidal capabilities (*59, 60*). Thus DC serves as an ideal niche for *M.tb* to replicate in while being able to inhibit and delay the immune cascade required for the induction of adaptive Th1 responses (See **Figure 2**).

### 1.4. Delayed induction of *M.tb*-specific Th1 responses at the lung dLN

As previously discussed, the induction of anti-TB Th1 immunity is substantially delayed in comparison to other pulmonary infections; the earliest induction of Th1 responses is not observed until day 14 post infection and occurs at the lung dLN (*18, 61, 62*) (also see **Figure 1**). The migration of Ag-bearing DC to the dLN is required for the priming of Th1 immune

responses required for host defense against *M.tb*. One possible mechanism explaining such delayed T cell priming may be due to the slow growth of *M.tb* species in comparison to other bacteria, which would delay the acquisition of a sufficient antigenic threshold required by APC for efficient T cell priming. This mechanism was proposed under the observation that the initiation of T cell priming correlated with the emergence of a certain threshold of bacteria within the dLN (*61, 63*). As the doubling time of *M.tb* is very long, the inability to efficiently reach a certain antigenic threshold may serve to delay DC antigen acquisition, maturation, and migration to the dLN, leading to a delay in T cell priming (*63*). In contrast to *M.tb*, rapidly growing mycobacteria such as *M. smegmatis* and *M. abscessus* have been demonstrated to trigger a more rapid T cell response and are thus able to efficiently clear the infection (*64*).

Another mechanism has suggested that the relative virulence of *M.tb* may play a role in the impairment of antigen processing. It has been found that while highly virulent strains of *M.tb* have the ability to inhibit apoptosis and instead promote necrosis, attenuated *M.tb* strains tend to undergo apoptosis instead (*65, 66*). It has been suggested that the inhibition of apoptosis and the promotion of necrosis prevents efficient processing of antigen, which is largely held as the primary reason for delayed T cell priming (*36, 67, 68*). However, evidence within the field and our own observations suggest that the relative virulence of *M.tb* may not be the major factor behind delayed T cell priming, as the kinetics of T cell activation following both virulent and attenuated *M.tb* are remarkably similar (*61, 62*). As such, other factors may be contributing towards delayed T cell priming.

*M.tb* has also been demonstrated to directly inhibit processes within DC involved in antigen presentation including the downregulation of MHC-II expression as well as blunting antigen processing (*69, 70*). In addition, mannose-capped lipoarabinomannan (manLAM) is a

PRR agonist expressed by *M.tb* that specifically binds to DC-SIGN expressed by DC, and has been demonstrated to inhibit the maturation of these cells (*71*). However, as recent studies have highlighted the heterogeneity of DC within the lung itself (discussed later in the thesis), it is not clear if all DC subsets are affected by *M.tb* in a similar fashion, and whether they all contribute equally to Th1 activation following pulmonary *M.tb* infection.

1.4.1. The role of IL-10 in the inhibition of anti-TB Th1 activation

*M.tb* has been demonstrated to be directly involved in immune suppression, whereby the induction of interleukin 10 (IL-10) by *M.tb* has been found to suppress macrophage activation by $CD4^+$ T cells (*72*, *73*). A well characterized regulatory cytokine, IL-10 signals through a receptor complex consisting of two subunits: IL-10R1 induced on stimulated haematopoietic cells, and IL-10R2 which is constitutively expressed on most cells (*74*). These receptor subunits signal through the Janus-kinase (JAK) and signal transducer and activator of transcription (STAT) pathway upon binding of IL-10, and cumulate in the phosphorylation and activation of STAT3 (*74*). Although there are other STATs that are also activated by this signaling cascade, it has been demonstrated that only STAT3 is required for the inhibitory effects of IL-10 (*75*, *76*).

IL-10 is produced by both myeloid cells and T cells, and a plethora of TLR agonists have been found to induce the expression of IL-10 including several *M.tb* antigens (*77*). Although T cells are able to produce IL-10 (*78*), DC are also an importance source of IL-10 (*79*). IL-10 has been found to inhibit many processes involved in T cell activation including migration of DC to the dLN (*80*) and antigen presentation via downregulation of MHC-II (*74*), and thus may play a direct role in inhibiting T cell priming at the dLN during T cell priming. However, whether all DC populations have similar capabilities in producing IL-10 is not clear. Nevertheless, we and others have demonstrated that IL-10 knockout animals display an accelerated T cell activation

kinetics following pulmonary *M.tb* infection (*81-83*). As such, the induction of IL-10 by *M.tb* may serve as an immune evasion mechanism by *M.tb* to inhibit Th1 activation.

Although the mechanism by which *M.tb* induces the expression of IL-10 is not clear, there is evidence to suggest that type I interferon may be involved. Several hypervirulent *M.tb* strains are strongly associated with the induction of type I interferon (*84, 85*), and recent *in vitro* evidence has demonstrated that the virulent signature of these clinical strains is due to the induction of IL-10 by type I interferon (*86*). There is also evidence to suggest that manLAM from *M.tb* is able to induce the production of IL-10 (*87*). Together this demonstrates that the induction of IL-10 by *M.tb* is likely a major mechanism contributing to delayed T cell activation. Although DC are known to produce IL-10, it is not clear whether all DC subsets are capable of producing IL-10 equally following pulmonary *M.tb* infection. Given that DC are critical in T cell priming, it is important to further investigate their role in T cell priming.

## 1.5. Dendritic cells are critical to the activation of Th1 responses

Central to Th1 induction are dendritic cells (DC), which were identified by Ralph Steinman in 1979 when his group was able to purify and identify these cells as a distinct population from macrophages and monocytes (*88*). Characterized by their long dendrites, these cells typically have very poor anti-bacterial capabilities (*60*) and are instead believed to be primarily responsible for alerting the immune system and linking the innate and adaptive immune compartment, particularly in the priming and activation of naïve T cells (*18, 89*). As previously discussed, maturation of these cells results in the upregulation of CCR7 and allows them to respond to chemotactic signals from the lung dLN, facilitating their migration. Once they arrive at the dLN, DC interact with the naïve T cells located within the dLN and initiate T cell priming. In particular, the arrival of these antigen-bearing DC are critical to T cell activation as

they provide the signals necessary for the generation of antigen specific T cells including (1) presentation of *M.tb* antigens to naïve T cells (2) co-stimulatory signals through molecules such as CD80/86 and (3) the secretion of T cell polarizing cytokines such as IL-12p70 (*90*). Upon recognition, the signals provided by the *M.tb*-loaded DC induces the naïve T cell to expand and polarize towards a Th1 phenotype.

As such, DC are critical to the activation of Th1 cells required for anti-TB immune responses. Although traditional definitions have broadly defined murine lung DC as $CD11c^{hi}MHCII^{hi}$ cells (*91*), recent studies have further refined this definition and expanded the lung DC population into several subsets. Under steady state conditions, lung DC can be broadly divided into plasmacytoid DC (pDC) and conventional DC (cDC), which can be further subdivided into $CD103^{+}$cDC and $CD11b^{+}$cDC (see **Figure 2**). In addition, under inflammatory conditions, monocyte-derived DC (moDC) can also be found. Although moDC have a different origin than other DC subsets, pDC and cDC are both derived from a common DC progenitor (CDP) found within the bone marrow, and is characterized by the expression of Fms-like tyrosine kinase 3 (FLT3) (*91-93*). FLT3 is crucial to pDC and cDC development as the lack of interaction with its ligand FLT3L leads to a total lack of both lineages within the animal, although specific transcription factors further distinguish these populations at later stages of development (*94, 95*). Finally, a resident DC population (rDC) has also been described at the secondary lymphoid organs (SLO), and although similar in function to cDC are believed to exclusively remain in SLOs (*89*). However, the relative role of each of the aforementioned DC subsets in T cell activation following pulmonary *M.tb* infection is not well described. Although human counterparts of these DC subsets have also been identified (*96*), the focus on this thesis

will be on the findings derived from murine models. Below is a brief summary of the roles identified for each DC subset in T cell activation (also see **Figure 3**).

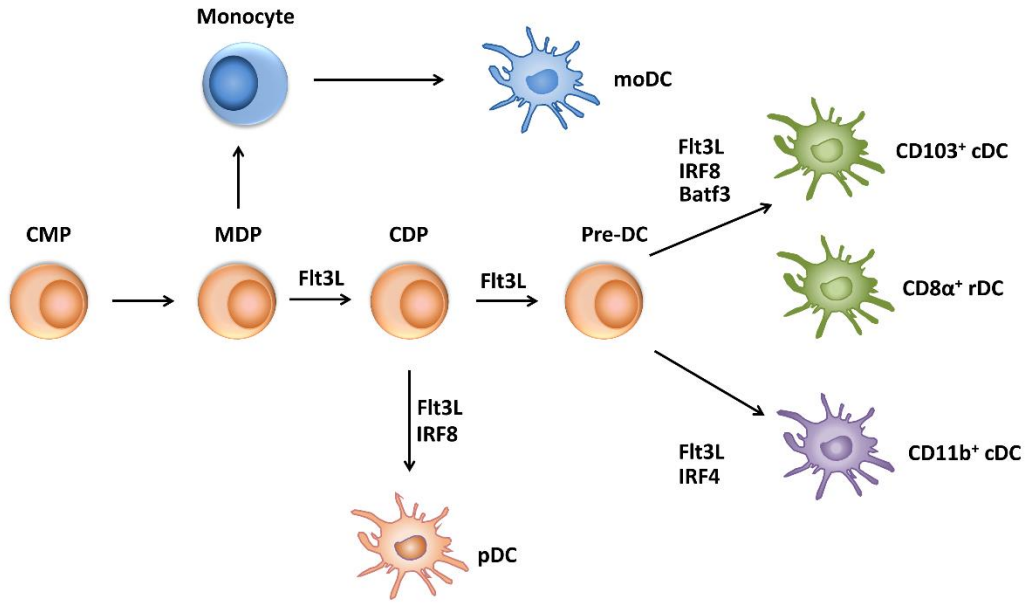



**Figure 3 – Differentiation of lung dendritic cell subsets is dependent on specific transcription factors**. Although all dendritic cells (DC) share a common progenitor, each subset has different requirements for their final differentiation. Within the lung and lung associated lymphoid organs, 4 major groups have been identified: (1) plasmacytoid DC (pDC) (2) conventional DCs (cDC), which can be sub-divided into $CD103^+$cDC and $CD11b^+$cDC (3) monocyte-derived DC (moDC) and (4) lymphoid resident $CD8\alpha^+$ DC (rDC).

1.5.1. The role of lung plasmacytoid DC in T cell activation

Traditionally, pDC have been associated with viral infections as they represent a major source of type I IFN (*97*). The specialization towards viral infections is associated with their high expression of TLR7 and TLR9, which allows them to robustly respond to viruses and self DNA (*98*). In mice, pDC are defined using CD11c, B220, bone marrow stromal antigen 2 (BST2) and Siglec-H, which differs from human pDC slightly as human pDC do not express any lineage-defining markers such as CD11c, CD19, CD14, CD16 and CD3 (*99*).

Unlike cDC, pDC exit the bone marrow and can enter secondary lymphoid organs directly through the high endothelial venules (HEVs) and not through the afferent lymphatics (*100*), although they also traffic to peripheral tissues as well. Specifically, their migration from the periphery to the dLN is dependent on the expression of CCR7 (*101*). Although pDC have been found to accumulate in the lymph nodes (*102*), the ability of pDC to present antigens and prime T cells remains somewhat controversial. Currently, the consensus is that pDC are capable of antigen presentation to both $CD4^+$ and $CD8^+$ T cells, albeit less efficiently than conventional DC populations (*103, 104*). Although it is not clear whether they are directly involved in T cell activation following pulmonary *M.tb* infection, the expression of PRR such as TLR2 (*105*) would allow them to respond to *M.tb* antigens. Furthermore, given that the increased virulent nature of many clinical *M.tb* strains is associated with a type I IFN signature (*84, 85*), it is likely that pDC play a role in host response to *M.tb*.

1.5.2. The role of conventional DC subsets in T cell activation

Although it was previously thought that pDC shared the same progenitor as other DC subsets (*106*), a pre-DC population that was able to differentiate into conventional DC (cDC) but not pDC was recently identified (*107*). These pre-DC were found to differentiate into two distinct subsets: a Batf3-dependent $CD103^+$cDC subset and a IRF4-dependent $CD11b^+$cDC

subset. Recently the use of XCR1 and signal-regulatory protein alpha (Sirpα) have also been used to more specifically identify CD103$^+$cDC and CD11b$^+$cDC respectively (*89*). The expression of distinct phenotypic markers and the requirement of specific transcription factors (Batf3 and IRF4) would suggest that these two cDC subsets are phenotypically and developmentally distinct from each other. However, there is ample evidence demonstrating that CD103$^+$cDC and CD11b$^+$cDC are functionally distinct from each other as well.

CD103$^+$cDC can be found within the lung epithelium, where the expression of CD103 on these DC allows them to associate with the lung endothelium via its interaction with E-Cadherin (*108*, *109*). In contrast, CD11b$^+$cDC are found within the lung lamina propria (*110*). Although the differences in anatomical location may suggest a difference in their relative ability for antigen uptake, both populations have been demonstrated to efficiently acquire and delivery antigens to the dLN depending on the model used (*56*, *69*, *111-114*). This suggests that the differences in the efficiency of antigen acquisition and delivery between CD103$^+$cDC and CD11b$^+$cDC may not be due to their relative location but rather a differential requirement for certain DC subsets in different infection models.

The migration of both CD103$^+$cDC and CD11b$^+$cDC subsets from the periphery to the dLN are also dependent on the expression of CCR7 (*101*). Specifically, the production of IL-12p40 is critically important for the expression of CCR7, as DC from IL-12p40 deficient animals fail to upregulate CCR7 and migrate to the dLN (*58*). However, the dominant cDC subset responsible for antigen delivery appears to vary depending on the pathogen involved. For example, following house dust mite (HDM) exposure, CD11b$^+$cDC and moDC were primarily responsible for the delivery of antigen to the dLN, with CD103$^+$cDC only participating at the highest dose of exposure (*112*). Following influenza infection, although CD11b$^+$cDC were more

efficient in the uptake of virus in the lung, these cells migrated poorly to the dLN. In contrast, $CD103^+$cDC were able to efficiently load viral antigens and migrate to the dLN (*115*). Although this question is not as clearly addressed following pulmonary *M.tb* infection, there is evidence to suggest that $CD11b^+$cDC may be more efficient in antigen delivery to the dLN (*69*).

Following their entrance into the dLN, both DC subsets present antigens at the dLN. Both DC participate in the presentation of antigens to naive $CD4^+$ and $CD8^+$ T cells present, and this has been extensively demonstrated in various models. During HDM exposure $CD11b^+$cDC and moDC (discussed below) but not $CD103^+$cDC were required for priming of HDM-specific $CD4^+$ T cells (*112*). In contrast, $CD103^+$cDC have been shown to be important in mediating cross-presentation of antigen and priming of $CD8^+$ T cells (*111*). However, another study has demonstrated that at the peak of infection $CD11b^+$cDC dominate $CD8^+$ T cell activation (*113*). During RSV infection both $CD103^+$cDC and $CD11b^+$cDC migrate to the dLN and present antigen equally well (*116*). Finally, there is evidence to suggest that $CD103^+$cDC are important for the induction of $CD4^+$ regulatory T cells ($T_{reg}$) through the production of TGF-β and retinoic acid (*117*), and has been demonstrated to be critical in preventing excess pathology following *M.tb* infection (*118*). The seemingly contradicting data demonstrates that the contribution of both $CD103^+$cDC and $CD11b^+$cDC subset to $CD4^+$ and $CD8^+$ T cell activation is also very pathogen dependent and cannot be generalized.

1.5.3. The role of resident DC subsets in T cell activation

Unlike migratory cDC, resident DC (rDC) differentiate and spend their entire lives in the SLO (*96*), which include the lung dLN. In the mouse, these cells have traditionally been differentiated based on a relatively lower expression of CD11c and MHC-II relative to cDC (*113*). However, more recently, several groups have differentiated rDC into two major

populations based on the expression of CD8α and CD4, although the use of CD4 as a distinguishing marker is somewhat controversial (*96*).

$CD8\alpha^+$ DC are also dependent on Batf3 for their final differentiation, demonstrating that they are of a similar lineage as $CD103^+$cDC (*119, 120*). Although not much is known regarding their role in pulmonary infections, a deficiency in these cells have been implicated to be correlated to a loss of $CD8^+$ T cell responses against West Nile virus (*119*). Furthermore, they have also been implicated as being critical for the production of IL-12 that is critical in the control of *Leishmania major* infection (*120*). Even less is known about the $CD4^+$ resident DC due to the difficulty in differentiating these cells from IRF4-dependent $CD11b^+$ cDC as well as moDC, as all three populations express CD11b (*96*). Whether these cells are involved in T cell priming following pulmonary *M.tb* infection has not been investigated.

1.5.4. The role of inflammatory monocytes and monocyte-derived DC in T cell activation

Monocytes develop in the bone marrow and remain in circulation until they are recruited to the periphery during an inflammatory response, where they differentiate into macrophages as well as DC (*121, 122*). These monocyte derived DC (moDC) are phenotypically very similar to $CD11b^+$cDC in that they share expression of CD11b, CD11c and MHCII expression, and both are Flt3 dependent (*106*). Currently it is still very difficult to differentiate between $CD11b^+$cDC which derive from the common DC progenitor (CDP) and the moDC which arise from a monocyte precursor, although there have been recent attempts to separate the two populations (*106, 112*). Traditional approaches have attempted to use the expression of Ly6C in conjunction with $CD11c^+$, $CD11b^+$ and MHC-II in order to separate moDC from $CD11b^+$cDC. However, given that moDC quickly downregulate Ly6C upon activation it is not a reliable marker (*96*). A

study by Plantinga *et al* has suggested the use of CD64 and Fc epsilon receptor as a way to separate moDC and CD11b$^{+}$cDC (*112*).

moDC are recruited to the lung following respiratory viral infection as well as allergen exposure (*123, 124*), and have been shown to be important for the delivery of antigen to the dLN (*112, 125*). In regards to antigen delivery and presentation following pulmonary *M.tb* infection there has been some suggestion that these cells are important for the delivery but not the presentation of *M.tb* antigens at the dLN (*69, 126*). Following HDM exposure, moDC along with CD11b$^{+}$cDC were important for antigen delivery and presentation to CD4$^{+}$ T cells (*112*). However, given the prior difficulties in distinguishing between conventional CD11b$^{+}$cDC and moDC further studies will be required to understand the role of moDC in T cell activation.

Collectively, the above data demonstrates that although various DC subsets are present in the lung and lung dLN, their relative contribution to T cell activation is very pathogen dependent and cannot be easily generalized between models. Given the heterogeneity that exist it is important to understand their relative role in T cell activation following pulmonary *M.tb* infection. However, as CD11b$^{+}$cDC and CD103$^{+}$cDC have been demonstrated to be both critical towards antigen delivery and antigen presentation, the focus of this thesis will be on these two DC subsets.

### 1.6. Delayed recruitment of T cells back to the lung leads to impaired control of *M.tb*

Despite the delay in T cell activation, by 14 days post infection the host eventually generates a Th1 immune response against *M.tb*. Upon activation in the dLN, these antigen-specific T cells expand in numbers, upregulate CD44 and downregulate CD62L (*127*) and migrate back to the site of infection at the lung between 15 to 21 days post infection. However, whether this delay in the migration of antigen-specific T cells back to the lung is due to a delay

in T cell priming or to a further impairment in the recruitment of T cells back to the lung is not clear (see **Figure 2**). Upon their arrival, these activated T cells interact with the infected macrophages, where the secretion of factors such as IFN-γ and TNF-α further activate infected macrophages in order to trigger key immune processes such as nitric oxide production and phagolysosome fusion essential for bacterial clearance (*36*). However, the delayed arrival of Th1 responses to the lung provides *M.tb* with a window in which it is able to replicate relatively unchecked. As such, despite the eventual arrival of Th1 responses the host is unable to effectively clear the bacteria and is at best only able to contain it.

## 1.7. Acceleration of Th1 immunity remains a goal of modern TB vaccine design

Despite the inability of natural host Th1 immunity to effectively eliminate *M.tb*, activation of infected macrophages by Th1 cells remains a critical aspect of anti-TB host defence. It has been proposed that the inability of natural host Th1 immunity to adequately protect against pulmonary *M.tb* infection is due to the delay in which this response arrives at the lung. As such, a goal of modern TB vaccine design for many years has revolved around the induction and acceleration of a robust anti-TB Th1 immunity. Given that the long history of BCG usage (*128*), the goal of many vaccine platforms involves boosting existing Th1 responses in BCG vaccinated individuals (*9*). In particular, our lab has demonstrated that the use of human adenovirus-based TB vaccine is an effective means to enhance existing BCG responses in previously vaccinated hosts (*129*).

## 1.8. AdHuAg85A – An example of a robust mucosal anti-TB vaccine platform

Adenoviruses are double-stranded DNA viruses with genomes that are approximately 34kbp in length. The adenovirus genome encodes a set of early transcription genes involved in gene regulation and replication (termed E1, E3, E4, and E5), and a set of late genes which primarily encode for structural genes (termed L1 through L5) (*130*). Given our extensive

understanding (and ease of manipulation) of the adenoviral genome, adenoviruses have been widely used as vectors for gene transfer as well as for vaccines. In fact, Adenoviruses remain the most widely used viral vectors for vaccine design given their excellent safety record (due to our ability to manipulate their genome) and their ability to drive robust adaptive T cell responses. In particular, human adenovirus serotype 5 (AdHu5) is the most widely used vector for vaccine design since they are proven to have an excellent safety record (*131*), are highly immunogenic, and are able to induce both CD8 and to a less extent CD4 T cell responses (*132, 133*). One of the most thoroughly characterized AdHu5 viral-vectored TB vaccines expresses the *M.tb* immune dominant antigen, Antigen 85A (hereby referred to as AdHuAg85A). Extensive pre-clinical studies in numerous animal models have demonstrated that this vaccine induces long lasting IFN-$\gamma^+$ TNF-$\alpha^+$ $CD8^+$ T cells, and to a lesser extent $CD4^+$ T-cell responses (*134, 135*) . Furthermore, a recently completed clinical trial has further demonstrated the immunogenicity of the vaccine where it was able to induce polyfunctional $CD4^+$ and $CD8^+$ T cell responses in BCG immunized human volunteers (*129*).

However, our preclinical data demonstrates a critical facet in vaccine design and implementation: the effectiveness of AdHuAg85A is dependent on the route of vaccination. Specifically, our previous studies demonstrate that respiratory mucosal, but not parenteral, vaccination with this vaccines is able to induce long lasting protection against *M.tb* infection *(134, 136)*. Until recently, it was believed that this was due to the ability of respiratory mucosal vaccination to induce a population of antigen specific T cells both in the airway and the lung interstitium (*137*). However, as intramuscular parenteral vaccination was also able to induce a population of *M.tb*-specific T cells at the lung (*134*), this did not seem a likely explanation.

The view that the presence of TB-reactive T cells in the lung is critical for protection ascends from prior consideration of the lung as a single compartment. The discrimination of the lung into multiple tissue compartments has led to a better understanding towards the anatomical distribution of immune responses in the lung. In addition to the airway and interstitium, the lung is also comprised of a dense network of pulmonary capillaries that forms a third compartment in the lung (*137*). The recent development of intravascular staining (*138*) has allowed for the identification of T cells in the lung interstitium vs T cells that remain within the lung vasculature. Using this technique, it was found that although both intranasal and intramuscular vaccination are able to induce *M.tb*-specific T cells at the lung, T cells generated following intranasal vaccination are found primarily in the interstitium and airway. This is in direct contrast to intramuscular vaccination, which induces T cells that are restricted to the lung vasculature (*137*). This demonstrates that only respiratory mucosal vaccination induces a bona fide antigen-specific T cell response in the lung.

## 1.9. Modulation of the local innate compartment by respiratory mucosal vaccination

Although it has been shown that mucosal vaccination is able to drive Ag-specific T cell responses to the airway, the factors that drive T cell entry into the lung airway and interstitium following respiratory mucosal vaccination remains unclear. However, it is well established that the innate immune compartment plays a critical role in the induction and instruction of adaptive T cell responses. In particular, it has been suggested that APC at peripheral sites are critical for directing T cells to localize towards a specific tissue compartments (*139*). As such, the superiority of respiratory mucosal vaccination may also be attributed to the ability of the viral vector to activate innate signals in the local environment. Adenoviruses are highly immunogenic, and AdHu5 in particular has been demonstrated to induce various pro-inflammatory cytokines

such as TNF-α, IL-6 and IL-12 (*140*, *141*). However, only respiratory mucosal vaccination would be able to directly modulate the local lung innate immune compartment. Given that we and others have identified various points in the immune cascade at which *M.tb* either directly or indirectly inhibits the innate immune response (*28*, *78*, *142*) (also see **Figure 4**), modulation of the lung innate immune compartment may represent a potential method to further enhance T cell responses and improve anti-TB immunity.

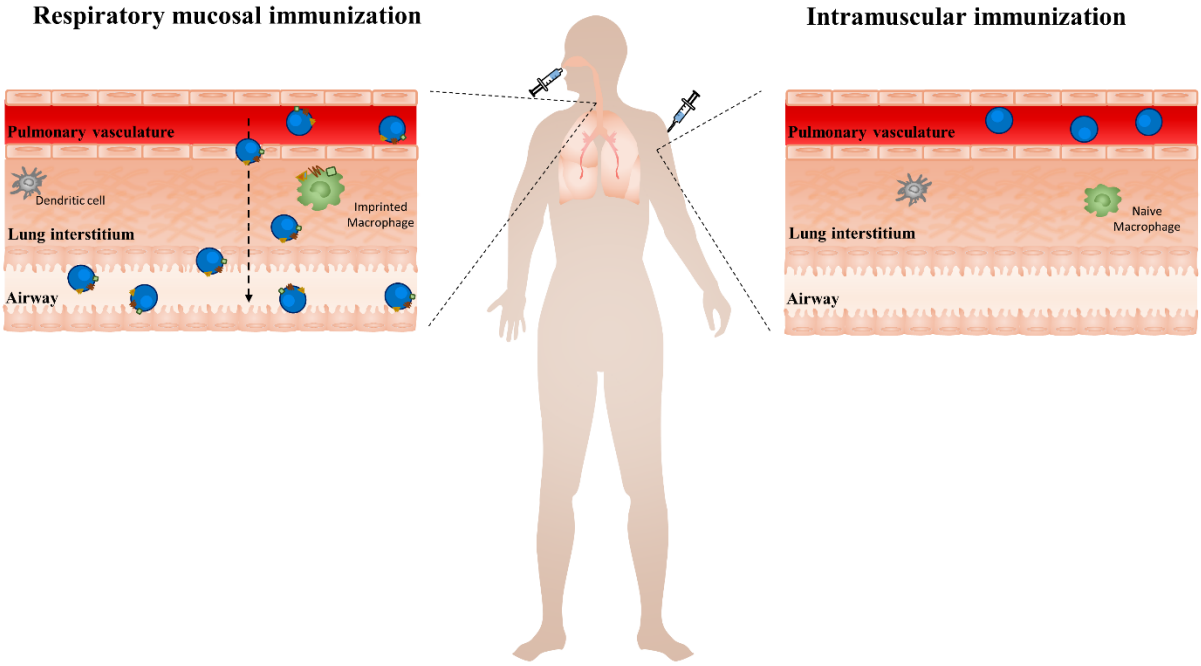



**Figure 4 – Modulation of the host innate compartment by respiratory mucosal AdHuAg85A vaccination**. The superior protection following respiratory mucosal vaccination with AdHuAg85A is traditionally associated with the presence of Ag-specific T cells within the lung airway and interstitium. However, respiratory mucosal vaccination also results in the modulation of the lung local innate compartment and potentially enhances the functionality of these cells.

However, the innate immune compartment has largely been overlooked in the design of novel TB vaccines. Although previous efforts have focused on the induction of an robust Th1 response as a direct correlate to protective immunity against TB (*18, 128*), we have demonstrated that the effects a vaccine has on the innate immune compartment also plays a major role in determining vaccine efficacy independent of the T cell responses elicited. We have previously demonstrated that despite inducing higher antigen-specific T cell responses, a vesicular stomatitis virus (VSV)-based TB vaccine expressing Ag85A (VSVAg85A) had poorer protective efficacy than AdHuAg85A. It was found that this is related to the induction of type I IFN by VSVAg85A, which induced the production of IL-10 and dampened the killing capacity of the infected lung macrophages (*143*). In contrast, AdHuAg85A did not lead to the induction of type I IFN. This data demonstrates that in addition to evaluating the magnitude and breadth of adaptive immunity, how a vaccine formulation affects both the local and systemic innate immune compartment should also be considered.

Furthermore, there is also evidence suggesting that the innate compartment contributes to protection against *M.tb* independently of adaptive T cell responses. The earliest evidence for this stems from a study of a TB outbreak on a US naval ship where they found 13 individuals that remained protected despite the absence of *M.tb*-specific T cell immunity for at least 6 months post exposure (*144*). Given the enclosed nature of a typical US naval ship, it is unlikely that they were never exposed to *M.tb*. As such, a more likely explanation points to the fact that an adaptive immune response was not needed because the innate response itself was highly effective and sufficient for protection. This has since been observed in many individuals who live in constant exposure (ie. household contact) of *M.tb* yet never demonstrated the induction of anti-TB T cell responses or signs of infection (*2*). This phenomenon has recently been described in literature as

early clearance of *M.tb* (*145*) (also see **Figure 1**), and suggests an independent role in protection for the innate immune compartment.

Further evidence of T cell-independent protection has been demonstrated using BCG. Although it is not clear whether BCG-mediated protection against *M.tb* involves the modulation of the innate immune compartment, there is substantial evidence that BCG is able to provide protection against heterologous infections in a T cell independent manner. The first evidence came from a Swedish study done in 1931 demonstrating that children vaccinated with BCG were protected against non-TB infections (*146*). This has also been demonstrated in a SCID mouse model using *Candida albicans* infection, which are extremely susceptible to *Candida albicans* infection. However, despite using a vaccine derived from an unrelated pathogen, these mice were protected if vaccinated with BCG prior to infection with *Candida albicans* (*147*). Furthermore, BCG has been demonstrated to increase responses in human monocytes and NK cells through epigenetic modifications (*147, 148*). This "imprinted" phenotype has also been demonstrated in macrophages following lipopolysaccharide (LPS) exposure (*149*). Given the immunogenic nature of viral vectored vaccines, it is interesting to speculate whether mucosal delivery of a vaccine vector such as AdHuAg85A would also result in the imprinting of the local innate immune compartment.

## 1.10. Conclusion

Until recently, the major direction of research towards the generation of host immunity against *M.tb* has been focused primarily on the induction of a robust Th1 response at the site of infection. While our understanding towards the role of Th1 response against *M.tb* has greatly improved over the last 20 years, our understanding of the role of the innate immune compartment in the generation of anti-TB immunity has not progressed in parallel. As previously discussed,

the induction of Th1 immunity is critically dependent on a robust innate immune response, specifically the innate mononuclear phagocytes. However, in addition to indirectly affecting anti-TB immunity through the induction of Th1 responses, there is also evidence to suggest that these cells are also able to directly contribute to the elimination of *M.tb* even in the absence of Th1 responses. As such, an increased understanding of how these cells contribute to the generation of host defence against *M.tb* will provide us with new tools to enhance and develop prophylactic and therapeutic interventions for the control and elimination of TB.

# Chapter 2. Central hypothesis and objectives

## 2.1. Rationale:

*Mycobacterium tuberculosis*, the causative agent of pulmonary tuberculosis, remains one of the leading causes of infectious disease-based death worldwide. Protective immunity against *M.tb* is dependent on the induction of Th1 responses, and current vaccine strategies have been focused on enhancing this aspect of host immunity. However, our lack of understanding towards host immune responses against *M.tb* has hindered our ability to develop new vaccines towards this goal. One aspect of host immunity that remains poorly understood is the delayed induction of Th1 responses, which impairs the ability of the host to control *M.tb* allows the bacteria to establish a foothold in the lung. Although it has been well recognized that the host innate immune response is critical to **the generation of Th1 immunity**, the specific factors contributing to delayed Th1 activation has not been well defined. As such, we seek to determine: **what are the defects within the innate compartment that contributes towards delayed Th1 priming following pulmonary *M.tb* infection**?

## 2.2. Central hypothesis:

We hypothesize that there exist **defects in the innate compartment** following pulmonary *M.tb* infection which contributes to delayed T cell activation. We believe that understanding of these factors will **aid in the development of improved vaccine strategies**. To demonstrate this, we seek to address the following objectives:

## 2.3. Objectives:

1. To determine the impact of the early innate inflammatory response on the generation of Th1 responses following pulmonary *M.tb* infection.

2. To investigate the relative contribution of specific pulmonary DC subsets in the induction of Th1 responses following pulmonary *M.tb* infection.

3. To determine the relative contribution of innate and adaptive immune responses in the early phase of *M.tb* infection in respiratory AdHuAg85A immunized hosts.

# Chapter 3. What is the impact of the host early innate inflammatory response on the generation of Th1 responses following pulmonary *M.tb* infection?

**Title of Manuscript**: Restoration of innate immune activation accelerates Th1 priming and protection following pulmonary mycobacterial infection

**Rocky Lai**, Mangalakumari Jeyanathan, Christopher R. Shaler, Daniela Damjanovic, Amandeep Khera, Carly Horvath, Ali A. Ashkar and Zhou Xing

Published in Eur. J. Immunol, 44(5):1375-86.

Published in the *European Journal of Immunology*

In this chapter, we examine whether the early innate inflammatory response plays a role in delayed T cell activation following pulmonary *M.tb* infection. Although it is understood that the early innate inflammatory response is critical for the downstream induction of adaptive T cell immunity, it is not clear whether any deficiencies in this compartment is responsible for the delayed induction of Th1 responses. Furthermore, given this dependency we also wanted to determine whether modulation of this immune compartment can accelerate the downstream immune cascade and enhance host anti-*M.tb* immunity.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

European Journal of Immunology

# Restoration of innate immune activation accelerates Th1-cell priming and protection following pulmonary mycobacterial infection

*Rocky Lai, Mangalakumari Jeyanathan, Christopher R. Shaler, Daniela Damjanovic, Amandeep Khera, Carly Horvath, Ali A. Ashkar and Zhou Xing*

McMaster Immunology Research Centre, M. G. DeGroote Institute for Infectious Disease Research, and Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada

**The immune mechanisms underlying delayed induction of Th1-type immunity in the lungs following pulmonary mycobacterial infection remain poorly understood. We have herein investigated the underlying immune mechanisms for such delayed responses and whether a selected innate immune-modulating strategy can accelerate Th1-type responses. We have found that, in the early stage of pulmonary infection with attenuated *Mycobacterium tuberculosis* (*M.tb* H37Ra), the levels of infection in the lung continue to increase logarithmically until days 14 and 21 postinfection in C57BL/6 mice. The activation of innate immune responses, particularly DCs, in the lung is delayed. This results in a delay in the subsequent downstream immune responses including the migration of antigen-bearing DCs to the draining lymph node (dLN), the Th1-cell priming in dLN, and the recruitment of Th1 cells to the lung. However, single lung mucosal exposure to the TLR agonist FimH postinfection is able to accelerate protective Th1-type immunity via facilitating DC migration to the lung and draining lymph nodes, enhancing DC antigen presentation and Th1-cell priming. These findings hold implications for the development of immunotherapeutic and vaccination strategies and suggest that enhancement of early innate immune activation is a viable option for improving Th1-type immunity against pulmonary mycobacterial diseases.**

**Keywords:** DCs · Delayed Th1 immunity · FimH · Mycobacterium · Tuberculosis

Additional supporting information may be found in the online version of this article at the publisher's web-site

## Introduction

Pulmonary mycobacterial infection is mainly caused by *Mycobacterium tuberculosis* (*M.tb*), the causative agent of tuberculosis (TB), and by other slow growing less virulent nontuberculous mycobacterial (NTM) species including *M. avium* and *M. kasasii*. The continuing high global incidence of and death due to tuberculosis infections [1] and the dramatic increase in pulmonary NTM disease globally over the past three decades [2] underscores the need to further understand the complex host immune responses to mycobacterial exposure in order to develop effective prophylactic and therapeutic strategies.

Protective immunity against mycobacterial infection requires the presence of a robust adaptive Th1-type response at the site of

*Correspondence:* Dr. Zhou Xing
e-mail: xingz@mcmaster.ca

infection. Upon exposure to the bacterium, mycobacteria is taken up by sentinel APCs such as DCs and delivered to the draining lymph node (dLN) [3, 4]. On arrival, these APCs present mycobacterial antigen to naive T cells within the dLN, thereby priming the antigen-specific T cells to a Th1 phenotype and stimulating their expansion [5]. The Th1 cells are then recruited back to the site of infection, and the secretion of Th1 cytokines such as IFN-γ by these cells is critical to activation of infected APCs to control bacterial growth [6]. However, one of the defining characteristics of host defense toward mycobacterial pathogens such as *M.tb* is the unusually long generation time required for the initiation of Th1 cell responses in the dLN and lung [7]. Such responses are not observed within the dLN until 10–14 days postinfection [5, 8–10] and are in direct contrast to the pace of T-cell responses to other intracellular bacterial pathogens such as *Legionella pneumophila* and *Mycobacterium abscessus* [11, 12]. This significant delay in the initiation of adaptive immune activation allows *M.tb* to establish a foothold in the lung and may account for the high incidence of latent *M.tb* infections around the world [7, 13, 14]. However, despite some recent progress [15–17], the immune mechanisms behind delayed Th1 immunity still remain poorly understood, and this has recently been identified as an important outstanding issue in the field of anti-mycobacterial immunity and TB vaccine research [7, 13, 14].

The relative virulence of *M.tb* has long been believed to play a role in delayed Th1-cell priming [7, 13–16]. It is less clear whether delayed Th1-cell priming also results from pulmonary NTM infection. Recent emerging evidence suggests that Th1-type immunity is delayed following infection not only by virulent *M.tb*H37Rv but also by attenuated slow-growing mycobacterial species *M.tb*H37Ra and *M. bovis* BCG [18–20]. These lines of evidence imply that other mechanisms may be at play. It has been well established that the initiation of adaptive immunity is critically dependent on the activation of early innate immune responses. There is abundant in vitro evidence to suggest that infection by slow-growing mycobacterial species inhibits the activation of innate macrophages and DCs [21–24]. However, whether early innate immune activation is impaired in vivo following pulmonary mycobacterial infection and how this activation relates to delayed Th1-type immunity is still poorly understood. Furthermore, little progress has been made toward identifying the immune-modulating strategies that can be utilized to accelerate Th1 immunity.

In this study, using a murine model of pulmonary mycobacterial infection we have investigated whether protective Th1-type immunity against pulmonary infection by *M.tb*H37Ra is delayed. We have dissected the early innate immune responses in the lung and dLN and investigated their relationship to Th1-cell activation and protection. We have found that, following pulmonary mycobacterial infection, the host fails to induce early innate immune and DC activation. This results in delayed Th1-cell activation and immune protection in the lung. However, lung mucosal delivery of the TLR agonist FimH is able to accelerate protective Th1-type immunity by restoring innate immune activation and enhancing DC migration and antigen presentation.

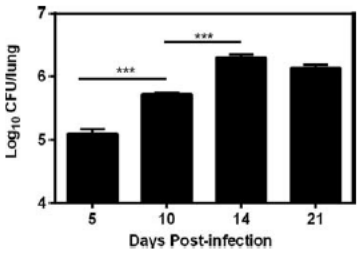



**Figure 1.** Control of pulmonary mycobacterial infection is delayed until days 14 and 21 postinfection. Lung homogenates from *M.tb*-infected mice were collected at various time points postinfection and subjected to a mycobacterial colony forming unit (CFU) assay to determine bacterial burden in the lungs. Data are expressed as mean + SEM of four mice per time point, representative of three independent experiments. ***$p < 0.001$ (one-way ANOVA with Tukey post-hoc analysis).

## Results

### Control of pulmonary mycobacterial infection is delayed until days 14–21 postinfection

It is well known that control of virulent *M.tb*H37Rv growth in the lung does not occur until days 14 and 21 postinfection [8] and during this period the level of infection continues to increase logarithmically. Previous studies have reported that, similar to that observed in *M.tb*H37Rv infection, there is a delay in bacterial control in the lung after *M.tb*H37Ra infection as well, as assessed by comparing bacterial burden at days 14 and 28 after infection [10, 18–20]. However, there is no information regarding the kinetics of infection within the first 14 days after pulmonary *M.tb*H37Ra infection. To address this issue, we assessed the levels of bacterial burden in the lung at early time points, including days 5, 10, 14, and 21 postinfection with *M.tb*H37Ra. We found that the levels of infection continued to increase logarithmically between days 5 and 14 and did not reach a plateau until days 21 (Fig. 1). These results indicate that, similar to *M.tb*H37Rv, the control of *M.tb*H37Ra infection by the host immune response is also significantly delayed.

### Delayed proinflammatory cytokine responses in the lung following pulmonary mycobacterial infection

The significantly delayed control of pulmonary mycobacterial infection described above suggests impaired innate immune responses in the early stage of infection. To begin investigating the potential immune mechanisms of delayed control of mycobacterial infection, we first examined the responses of proinflammatory cytokines and chemokines in the lung in the early stage of infection. We elected to focus on examining the levels of TNF-α, IL-12, MCP-1, keratinocyte-derived chemokine (KC), and IFN-γ, as these cytokines are involved in the development of anti-mycobacterial Th1 immunity [6]. The levels of TNF-α, IL-12, and MCP-1 were

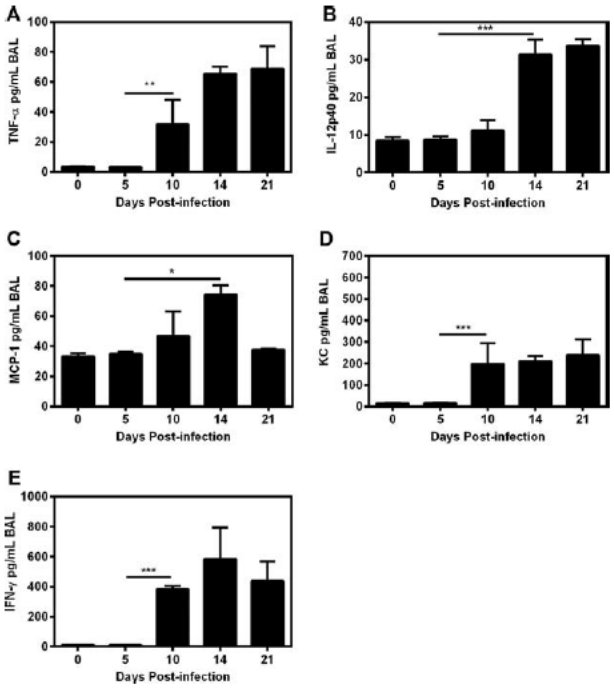



**Figure 2.** Delayed proinflammatory cytokine and chemokine responses in the lungs following pulmonary mycobacterial infection. Bronchioalveolar lavage (BAL) fluids were collected at various time points from the lungs of *M.tb*-infected mice and were assessed for concentrations of cytokines and chemokines. Lung levels of (A) TNF-α, (B) IL-12 p40, (C) MCP-1, (D) KC, and (E) IFN-γ were determined using a 22-plex Luminex Kit at the indicated time points. Data are expressed as mean + SEM of three mice per group/time point, representative of two independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one-way ANOVA with Tukey post-hoc analysis).

not significantly increased in the lung until day 14 postinfection (Fig. 2A–C). In comparison, the levels of KC and IFN-γ were not significantly increased until day 10 postinfection (Fig. 2D and E) and the levels of MIP-1α and IP-10 did not significantly increase until day 10 (data not shown). These data suggest that in general there is a significant delay in the upregulation of key proinflammatory cytokine responses in the lung following pulmonary mycobacterial infection.

## Delayed innate cellular immune responses in the lung following pulmonary mycobacterial infection

Given the delay in the upregulation of proinflammatory cytokine and chemokine responses in the lung, we next examined the kinetics of the recruitment of innate immune cells, particularly DCs, in the lung following pulmonary mycobacterial infection. DC are known to be critical to T cell priming in the local draining lymph node following *M.tb* infection [7, 13, 14]. By using a panel of surface markers, we specifically examined the responses of alveolar macrophages (AMs) ($AF^{+}CD11c^{+}$) and DCs ($AF^{-}CD11c^{+}MHCII^{+}$) in the lung (the gating strategy for each was defined in Supporting Information Fig. 1) [25, 26]. In keeping with the initially delayed proinflammatory cytokine and chemokine responses (Fig. 2), significantly increased AM responses were not observed until day 21 postinfection (Fig. 3A). Likewise, the responses of $CD11c^{+}MHCII^{+}$ DCs were not significantly increased until day 10 postinfection (Fig. 3B). Neutrophil responses were similarly delayed (Fig. 3C). Similar innate cellular responses were also seen in the bronchoalveolar lavage fluids (data not shown). These data together suggest that following pulmonary mycobacterial infection, there is a delay in proinflammatory cytokine and cellular responses in the lung.

## Delayed arrival of antigen-bearing DC in dLN is associated with delayed Th1-cell priming

As DCs are known to be critical to T cell priming in the local draining lymph node following *M.tb* infection [7, 13, 14], our data thus far suggest that significantly delayed initiation of innate immune responses in the lung may negatively affect DC migration from the site of infection to the local dLN and the subsequent Th1-cell priming, which could be an important downstream immune mechanism for delayed control of *M.tb*H37Ra infection in the lung. To this end, we investigated the timing of DC arrival at the dLN (gating strategy was defined in Supporting Information Fig. 2) [25, 26] and its relationship to the appearance of mycobacteria and antigen-specific Th1 cells in the dLN. We found that the significant arrival of $CD11c^{high}MHCII^{high}$ DCs at the dLN was not seen until day 10 postinfection (Fig. 4A). This coincided well with significant appearance of viable mycobacteria in the dLN (Fig. 4B).

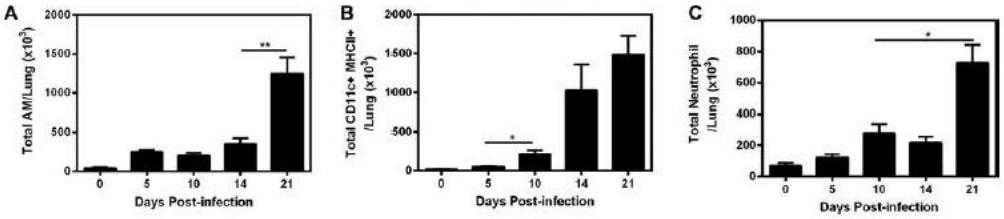



**Figure 3.** Delayed innate cellular immune responses in the lung following pulmonary mycobacterial infection. Mononuclear cells were isolated from the lungs of mice at various time point post-*M.tb* infection. The numbers of (A) alveolar macrophages (AM), (B) $CD11c^+MHCII^+$ DCs and (C) neutrophils in the lungs were determined using immunostaining and flow cytometry (see Supporting Information Fig. 1 for gating strategy). Data are expressed as mean + SEM of three mice per group/time point, representative of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA with Tukey post-hoc analysis).

To examine the relationship of DC arrival and myocbacterial appearance in the dLN to Th1 priming, we examined the kinetics of mycobacteial antigen-specific Th1-cell priming in the dLN. Significant Th1-cell priming was not detectable in the dLN until day 10 and its levels (both the frequencies and absolute cell numbers) continued to climb at days 14 and 21 postinfection (Fig. 4C). This agrees with previously published data [10, 18], and further suggests that delayed activation of innate immune cells, particularly DC, in the lung and the dLN are closely associated with delayed mycobacterial antigen-specific Th1-cell priming.

## Th1-type cellular responses in the lung following pulmonary mycobacterial infection

Following T cell priming at the dLN, activated T cells enter the circulation and are recruited to the site of infection [7]. We next examined whether the significantly delayed Th1-cell priming observed in our study led to retarded Th1-cell responses in the lung following pulmonary mycobacterial infection. Indeed, in keeping with delayed Th1 priming in the dLN (day 10) (Fig. 4C), the significantly increased recruitment of Th1 cells to the lung did

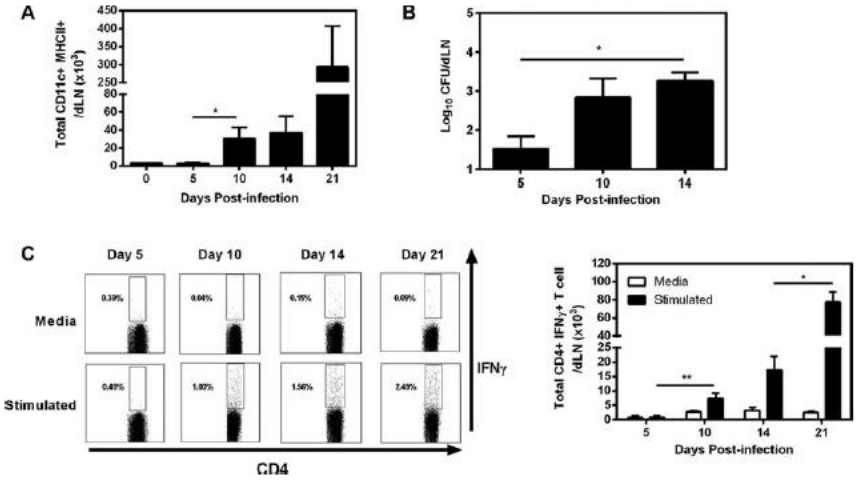



**Figure 4.** Delayed arrival of antigen-bearing DCs at the dLN is associated with delayed Th1-cell priming. (A) The dLN were collected at various time points post-*M.tb* infection. Mononuclear cells isolated from dLN were subjected to immunostaining and flow cytometry (see Supporting Information Fig. 2 for gating strategy) to determine the numbers of $CD11c^{high}MHCII^{high}$ DCs. (B) In a separate experiment, the dLN collected at various time points were also subjected to a mycobacterial CFU assay. (C) Some mononuclear cells isolated at various time points from dLN were subjected to antigen stimulation and immunostaining, intracellular cytokine staining and flow cytometry to quantify antigen-specific $CD4^+IFN\text{-}\gamma^+$ T cells. Dots plots are show of unstimulated (media) and stimulated samples at each time point and are representative of three independent experiments. Data in all graphs are expressed as mean + SEM of three mice per group/time point, representative of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA with Tukey post-hoc analysis or two-tailed student's *t*-test).

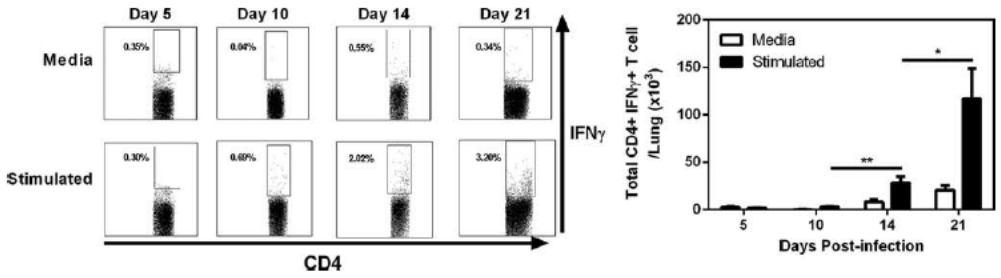



**Figure 5.** Th1-type cellular responses in the lung following pulmonary mycobacterial infection. Lung mononuclear cells were collected at various time points post-*M.tb* infection and subjected to antigen stimulation, immunostaining, and intracellular cytokine staining and flow cytometry to quantify antigen-specific $CD4^+IFN\text{-}\gamma^+$ T cells. Dots plots show unstimulated (media) and stimulated samples at each time point and are representative of three independent experiments. Data are expressed as mean + SEM of three mice per group/time point, representative of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ (two-tailed Student's *t*-test).

not occur until day 14 and the Th1 cells continued to increase in both frequency and absolute numbers by day 21 postinfection (Fig. 5). The above results together suggest that following pulmonary mycobacterial infection, the delayed activation of innate immune cells, particularly DCs, in the lung and dLN impedes Th1-cell priming and lung recruitment, which accounts for the lack of control of mycobacterial infection in the lung.

### Restoration of innate immune activation by intranasal FimH delivery following mycobacterial infection

Having demonstrated the lack of early innate immune activation in the early stage of pulmonary mycobacterial infection, we sought to investigate whether immunomodulatory strategies aimed at accelerating innate immune activation in the infected lung would improve downstream immunologic events. Certain TLR agonists are capable of innate immune activation and have been explored as immune adjuvant in vaccine formulations [27]. In this regard, we have recently identified the TLR4 agonist FimH, the adhesion portion of *Escherichia coli* type I fimbriae, to be a potent immune activator with a major effect on DC in the lungs [28], thus making it an attractive candidate immune modulator in our model. To this end, after the establishment of infection, mice were then inoculated intranasally once with FimH at day 2 postinfection. Proinflammatory cytokine and innate immune cellular responses were determined in the lung at days 5, 10, and 14 postinfection (Fig. 6). Compared with PBS-treated infected control mice, FimH exposure significantly increased the levels of proinflammatory cytokines TNF-α and IL-12 in the lung by day 5 and day 10 postinfection, respectively (Fig. 6A and B). In keeping with increased cytokine responses, the responses of AM and $CD11c^+MHCII^+$ DC were significantly increased in the lung at days 5 and 10 postinfection (Fig. 6D and E). Of note, FimH exposure did not significantly modulate neutrophilic responses to infection (Fig. 6C), reaffirming its effects on APCs. These results suggest that postinfection delivery of FimH is able to enhance early innate immune activation in the lung following pulmonary mycobacterial infection.

### Accelerated arrival of DC and Th1-cell priming at the dLN by single intranasal FimH delivery

Given the FimH-enhanced activation of innate immune responses, particularly on APCs in infected lungs, we determined the effect of FimH on the downstream immunologic events in the dLN. After treating the infected mice with FimH as described in Figure 6, we analyzed the DC responses and *M.tb*-carrying DCs in the dLN at days 5, 10, and 14 postinfection. Compared with PBS-treated infected control hosts, FimH exposure significantly increased $CD11c^{high}MHCII^{high}$ DCs in the dLN as early as at day 5 postinfection (Fig. 7A). The number of DCs in the dLN of FimH-treated animals remained significantly elevated over the control by day 10 postinfection (Fig. 7A). As a result of increased DC migration from the lung to the dLN, there was a markedly increased number of DCsin the dLN that contained viable mycobacteria at day 5 (Fig. 7B). Of importance, the enhanced arrival of *M.tb*-bearing DCs was found to lead to accelerate antigen-specific Th1-cell priming in the dLN at early time points (days 5 and 10) postinfection (Fig. 7C).

To further investigate the role of FimH-increased DCs in accelerated Th1-cell activation in the dLN following pulmonary mycobacterial infection, we used an in vivo Ag-presentation and T cell activation model, in which we adoptively transferred the same number of *M.tb* Ag85B-specific transgenic CD4 T cells (P25-Tg cells) into mice that were subsequently *M.tb*-infected and FimH-treated (or PBS-treated). We then examined the extent of transgenic T-cell activation (proliferation) within the dLN (Fig. 7D). Indeed, compared with the PBS-treated control animals, increased rates of active proliferation of antigen-specific transgenic T cells were observed in the dLN of FimH-exposed

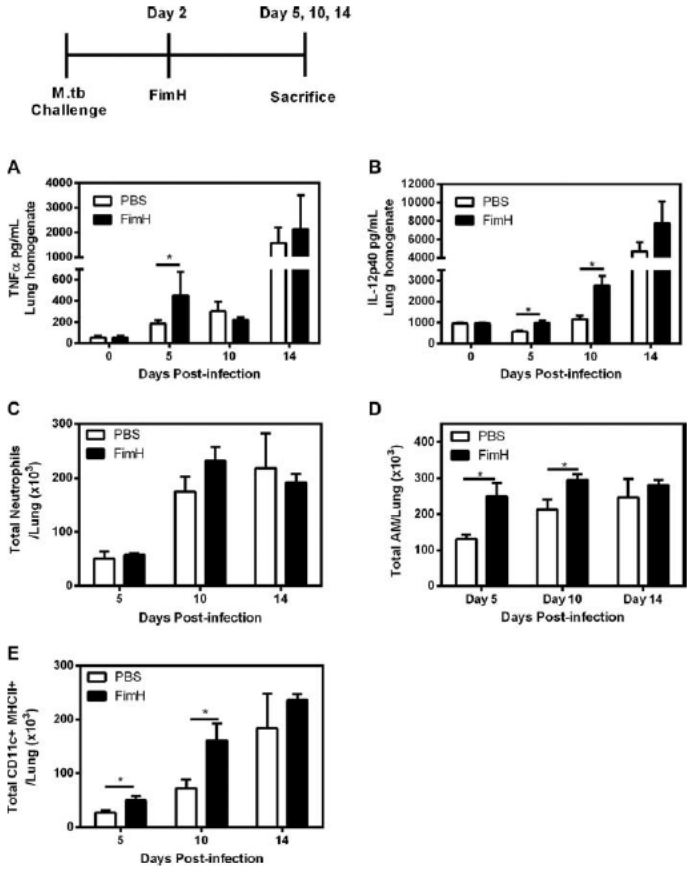



**Figure 6.** Acceleration of early innate immune activation by single intranasal delivery of the TLR4 ligand FimH following pulmonary mycobacterial infection. Mice were infected with *M.tb* and FimH was delivered intranasally at day 2 postinfection. Control mice received only PBS. Lung homogenates were prepared at various time points and were assessed by ELISA for concentrations of (A) TNF-α and (B) IL-12 p40. Mononuclear cells were isolated from enzymatically digested lungs at the indicated time points and subjected to immunostaining and flow cytometry to determine the number of (C) neutrophils, (D) alveolar macrophages (AM) and (E) $CD11c^+MHCII^+$ dendritic cells. Data are expressed as mean ± SEM of three mice per group/time point, representative of two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ (two-tailed Student's t-test).

hosts (Fig. 7D and E). The above results together indicate that FimH exposure accelerates Th1-cell activation via enhancing DC responses and functionality in the dLN.

## Accelerated DC and Th1-cell responses by FimH lead to improved control of mycobacterial infection

We next examined whether FimH-accelerated DC activation and T cell priming in the dLN led to increased Th1-cell responses in the lung at early time points following mycobacterial infection. Indeed, compared with PBS-treated control animals, the levels of antigen-specific Th1-cell responses in the lung were significantly increased both at days 10 and 14 postinfection (Fig. 8A) and as a result, the levels of immune protection were markedly increased at days 10 and 14 postinfection as indicated by about a 1-log reduction in the level of mycobacterial infection in the lung (Fig. 8B).

To further investigate the role of FimH-increased Th1-cell responses in enhanced bacterial control in the lung, mice were infected with *M.tb*H37Ra and treated once i.n. with FimH as described in Fig. 6 and then depleted of total T cells with anti-CD4 and -CD8 antibodies at day 5 postinfection (Fig. 8C). The control groups included FimH-treated mice receiving control IgG injection (FimH IgG), PBS-treated mice receiving anti-T cell antibodies (WT dep) and PBS-treated mice receiving control IgG (WT IgG). All of the mice were sacrificed and assessed for lung infection at day 10 postinfection. As expected, depletion of T cells in the WT dep group resulted in no significant changes in bacterial burden compared with the WT IgG group (Fig. 8C). On the other hand, FimH-treated mice receiving the control IgG (T cell-competent hosts) had significantly enhanced immune protection (reduced levels of infection) in the lung (Fig. 8C), consistent with the findings in Figure 8B. In contrast, depletion of T cells in FimH-treated hosts (FimH dep) resulted in a complete loss of FimH-enhanced

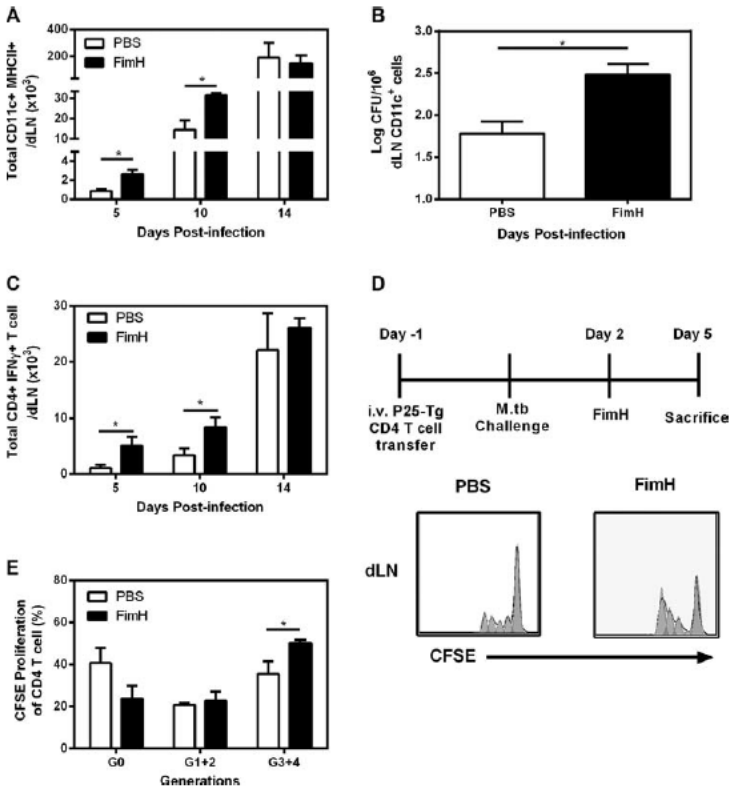



**Figure 7.** Accelerated arrival of antigen-bearing DCs and Th1-cell priming in the dLN upon single intranasal delivery of FimH following pulmonary *M.tb* infection. Mice were infected with *M.tb* and FimH was delivered intranasally at day 2 postinfection. Control mice received only PBS. (A) Mononuclear cells isolated from the dLN at the indicated time points were subjected to immunostaining and flow cytometry to determine the number of $CD11c^{high}MHCII^{high}$ DCs (see Supporting Information Fig. 2 for gating strategy). (B) Mycobacterial loads within the $CD11c^+$ DCs in the dLN were determined at day 5 postinfection using a CFU assay. (C) The number of antigen-specific $CD4^+IFN\text{-}\gamma^+$ T cells was determined in the dLN using immunostaining and intracellular cytokine staining and flow cytometry. (D) P25-Tg $CD4^+$ T cells isolated from P25-Tg Ag85B mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred intravenously into mice one day prior to *M.tb* infection. Proliferation of these cells in the lung dLNs was assessed using flow cytometry ($CFSE^+CD4^+$) at day 5 postinfection. Histograms are representative of two independent experiments. (E) Based on the relative CFSE dilution, proliferation (%) was quantified by separately comparing the P25-Tg $CD4^+$ T cells shown in (D) that underwent no proliferation (G0), 1–2 generations of proliferation (G1 + 2) and 3–4 generations of proliferation (G3 + 4). Data are expressed as mean + SEM of three mice per group/time point, representative of two independent experiments. $^*p < 0.05$ (two-tailed Student's *t*-test).

protection (Fig. 8C). These results suggest that FimH exposure increases early immune protection via DC activation-mediated acceleration of Th1-cell responses in the lung.

## Discussion

The poorly understood immune mechanism behind delayed Th1-type immunity following pulmonary mycobacterial infection has recently been identified as an important outstanding issue in the field of anti-TB immunity and vaccine development [7, 13, 14]. Although it is known that delayed Th1-type immunity in the lung is a characteristic of host defense against infection by slow growing and less virulent *M.tb* strain such as *M.tb*H37Ra [10], the upstream immunologic events are still poorly understood. In the current study, we have investigated the underlying immune mechanisms of delayed protective Th1-type immunity against *M.tb*H37Ra infection and to which extent this situation can be rectified by using a selected immune-modulating strategy. In agreement with previous studies [5, 8–10, 18], we find that the immune protection is significantly delayed as the level of infection in the lung continues to increase logarithmically until days 14 and 21 postinfection.

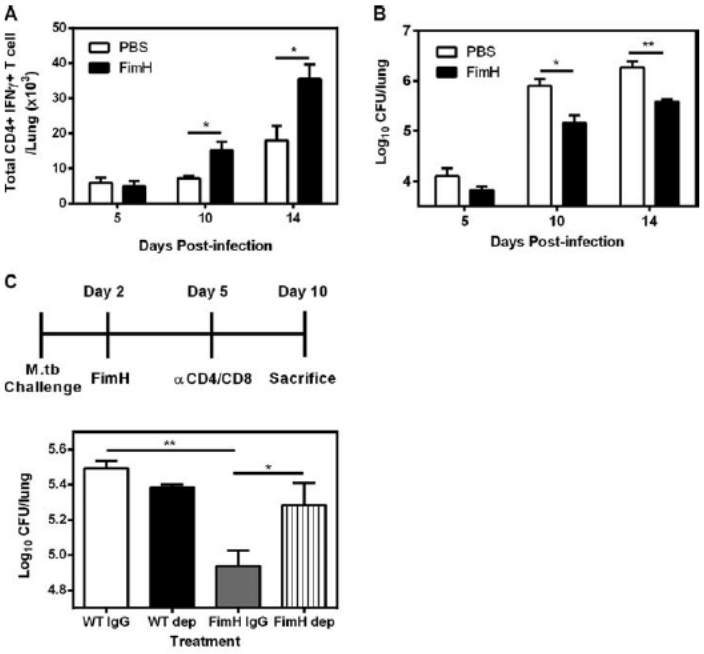



**Figure 8.** Accelerated DC activation and Th1-cell responses upon FimH exposure lead to improved control of pulmonary mycobacterial infection. Mice were infected with *M.tb* and FimH was delivered intranasally at day 2 postinfection. Control mice received only PBS. (A) Mononuclear cells were isolated from enzymatically digested lungs at the indicated time points postinfection and were subjected to immunostaining and flow cytometry to determine the number of $CD11c^+MHCII^+$ DCs (see Supporting Information Fig. 1 for gating strategy). (B) Lung homogenates collected at the indicated time points post-*M.tb* infection were subjected to a CFU assay to determine the bacterial burden in the lungs. (C) At day 5 postinfection (3 days post-FimH treatment), mice were given CD4/CD8-depleting antibodies or IgG control intraperitoneally. Mice were sacrificed at day 10 postinfection, and lung homogenates were subjected to a CFU assay in order to determine bacteria burden in the lungs. Data are expressed as mean + SEM of four mice per group, representative of two independent experiments. $^*p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA with Tukey post-hoc analysis or two-tailed Student's *t*-test).

We further find that in the early stage of infection, the activation of innate immune responses, particularly DCs, in the lung is delayed. This results in a delay in the downstream immune responses including the migration of antigen-bearing DCs to the dLN and DC-mediated Th1-type priming and subsequent Th1-cell recruitment to the lung. On the other hand, lung exposure to a TLR4 agonist FimH is able to accelerate protective Th1-type immunity in the early stages of infection by restoring early pro-inflammatory responses and DC activation and facilitating DC-mediated Th1-cell priming.

A critical aspect of the activation of innate immune responses following pulmonary *M.tb* infection is the recruitment and activation of key APC populations, particularly DCs. Although it was initially thought that alveolar macrophages represented the major target of infection by mycobacteria, recent findings have also identified DCs as a cellular target of mycobacterial infection [3, 21]. DCs have long been identified to be a major cell type involved in the delivery and presentation of antigen to naive T cells [8, 29] and as such represent a critical linkage between the innate and adaptive immune responses. Although previously published studies have provided ample evidence that slow-growing mycobacterial species are able to suppress innate macrophages and DC in vitro [21–24], it remains largely unclear whether this is the case in vivo and if so, what is its relationship to delayed Th1-type immunity. In this regard, our current study has identified the delayed activation of early innate DC responses in the lung and draining lymph nodes to be an important mechanism accounting for delayed protective Th1-type immunity. Thus, the lung deposition of a selected innate TLR ligand (FimH) particularly adept at APC activation is able to accelerate protective Th1-type immunity by facilitating DC migration to the lung and draining lymph nodes, thus enhancing DC antigen presentation and Th1-cell priming.

Some groups have previously attributed delayed T-cell priming to a lack of sufficient antigenic load during the early phase of *M.tb* infection [8, 30], which in turn has been suggested to be due to the virulence of *M.tb* itself [16, 31]. This notion is based on the observation that while the cells infected with attenuated *M.tb*H37Ra undergo apoptotic cell death pathways, virulent *M.tb*H37Rv infection tends to promote necrotic cell death, thus preventing the efficient generation of mycobacterial antigen and subsequently, delaying T-cell priming [15, 16, 32]. Our current findings support the notion that delayed Th1-type immunity is rather a trait of infection by the slow-growing mycobacterial species including *M.tb*H37Rv, *M.tb*H37Ra, and *M. bovis* BCG, independent of the relative virulence. This notion is in alignment with the findings from recently published studies [18, 19, 33]. Furthermore, a recent study has provided the evidence that delayed Th1-cell priming is

also independent of the antigenic load following *M.tb* infection [34].

With the delayed DC responses identified as central to delayed Th1-type immunity in our current study, we have also observed the blunted responses of proinflammatory cytokines and chemokines including TNF-α, IL-12 p40, and MCP-1 in early phases of pulmonary mycobacterial infection. This is in alignment with the recent finding that proinflammatory cytokine TNF-α production is blunted during the early phase of pulmonary *M. avium* infection [35]. While these cytokines play a multifunctional role in anti-TB immunity [6, 29, 36], we believe that lack of such cytokines is the main reason for retarded DC migration and activation both in the lung and draining lymph nodes. Indeed, IL-12 p40 has been described to play a critical role in mediating the migration of DCs to the dLN following *M.tb* infection [29]. Delayed innate immune activation following mycobacterial infection is likely attributed to the robust immune suppressive property of the mannose-capped lipoarabinomannan (manLAM) in the cell wall of slow-growing mycobacterial species such as *M.tb*H37Rv, *M.tb*H37Ra, and BCG [13].

Our finding that the mucosal exposure to the TLR ligand FimH accelerates protective Th1-type immunity further supports the importance of adequate early proinflammatory cytokine responses and DC activation. Although TLR agonists have often been used in experimental and clinical studies as an immune adjuvant [27], many are associated with significant side effects or toxicity [37, 38]. FimH represents a recently discovered TLR4 ligand with a great potential to be a safe and effective immune modulator, particularly amenable for mucosal application based on its selective effect on APCs [39]. Indeed, we have previously observed that when delivered to the respiratory tract, it induces robust anti-influenza innate immune responses with minimal structural damage in the lung [28]. We have also observed an enhanced inflammatory response upon delivery to the respiratory tract, resulting in enhanced recruitment of APCs to the lung. Of interest, in our current study we find FimH exposure to have a minimal effect on neutrophil responses in the lung. On the other hand, a recent study has suggested that neutrophils may also promote T cell activation by enhancing antigen acquisition of APCs through increased formation of apoptotic bodies containing mycobacterial antigen [16, 40]. Since human neutrophils exposed to type 1 fimbriated *E. coli* were found to have enhanced apoptosis [41], we cannot rule out that in vivo FimH administration may have increased neutrophil apoptosis, thus enhancing the antigen acquisition capabilities of DCs.

Our study thus highlights a potential for use of FimH as a therapeutic agent against TB or an immune adjuvant for subunit-based TB vaccines. Indeed, even administration of FimH at 2 days after infection helped control bacterial growth against *M.tb*H37Ra in naïve animals. It may also be considered for applications aimed at enhancing protection in parenteral BCG-vaccinated hosts, since we have recently found that BCG vaccination fails to control bacterial growth in the lung up to 14 days after either *M.tb*H37Ra or *M.tb*H37Rv infection [19]. Further investigation is required to evaluate the therapeutic effect of FimH treatment in models of *M.tb*H37Rv infection as the virulent *M.tb* may have differential innate immune-modulatory properties [18]. Moreover, FimH-mediated innate immune modulation strategies during pulmonary tuberculosis should be implemented with caution as imbalance in the innate immune cell activation may result in undesired immunopathology [42].

In summary, we have found that immune protection is significantly delayed following pulmonary mycobacterial infection and the delayed DC activation is central to delayed protective Th1-type immunity. We have further discovered that TLR agonist FimH-based immune-modulating activity is able to accelerate protective Th1-type immunity via its activating effect on DC migration and activation. These findings provide new mechanistic insights to the host-mycobacterium interaction and hold implications for developing immunotherapeutic and vaccination strategies against pulmonary mycobacterial diseases.

## Materials and methods

### Mice

Female C57BL/6 mice (6–8 weeks old) were purchased from Charles River (Wilmington, MA, USA). P25TCR-Tg mice (C57BL/6-Tg(H2-Kb-Tcra, -Tcrb)P25Ktk/J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in Level B rooms within the Central Animal Facility at McMaster University. All experiments were conducted in accordance with the McMaster University Animal Research Ethics Board.

### *Mycobacterium tuberculosis* preparation and infection

*M.tb*H37Ra was prepared for infection as previously described [19]. Mice were sacrificed at 5, 10, 14, or 21 days postinfection. Bacterial burden within the lung and lymph node was assessed at each time point as previously described [19].

### Intranasal administration of FimH

FimH, a component of the *E. coli* type I fimbriae and a potent TLR4 ligand, was expressed and purified as previously described [39]. Where indicated, mice received 10 μg of FimH in PBS i.n. 2 days postinfection.

### Mononuclear cell isolation and purification

Bronchioalveolar lavage (BAL) was performed on the lungs to isolate the airway luminal cells as previously described [19, 43]. Following lavage, the lungs were cut into small pieces and digested with 150 U of collagenase type I from Gibco (Grand Island, NY, USA) in Hanks Buffer for 1 h at 37°C with agitation. Lymph nodes were teased apart on a petri-dish with 23-gauge needles and digested with 1 μg/mL of Collagenase Type II from Worthington

(Lakewood, NJ, USA) in RPMI for 1 h at 37°C with agitation. The digested lungs and lymph nodes were then crushed through a 100-μm filter from BD Falcon (Franklin Lakes, NJ, USA) and lungs were treated with ACK Lysis buffer for 2 min to remove all Red Blood cells as previously described [19]. Cells were resuspended in RPMI supplemented with 10% FBS, 1% Pen-Strep and 1% L-Glutamine (cRPMI).

The purification of $CD11c^+$ cells and $CD4^+$ cells were performed as previously described [19] using MS column on the OctoMax separator. Purity was assessed using flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA).

### Cell surface and intracellular cytokine staining

T-cell responses were determined from mononuclear cells isolated from the BAL, lung, or lymph node as previously described [19, 44]. The following antibodies from BD Biosciences (San Jose, CA, USA) were used: CD3-V450, CD4-PE-Cy7, CD8a-allophycocyanin-Cy7, IFN-γ-allophycocyanin. Data were collected using the LSRII and FACSDiva software from BD Biosciences (San Jose, CA, USA) and analyzed using FlowJo Software from Treestar (Ashland, OR, USA).

Immunoprofiling of innate immune cells were also performed by staining for cell surface markers with the following antibodies: CD45-allophycocyanin-Cy7, CD11b-PE-Cy7, CD11c-allophycocyanin from BD Biosciences (San Jose, CA, USA); MHC-II (I-A/I-E)-Alexa Fluor 700 from eBiosciences (San Diego, CA, USA); Ly6C/G-Pacific Orange and Qdot800 from Invitrogen (Burlington, ON, Canada). Data were collected using the LSRII and FACSDiva software from BD Biosciences (San Jose, CA, USA) and analyzed using FlowJo Software from Treestar (Ashland, OR, USA).

### Cytokine and chemokine measurement

Cytokine and chemokine levels within the BAL fluids, lung, and lymph node homogenates were determined using a 22-plex Milliplex Mouse Cytokine/Chemokine Immunoassay Kit from Millipore (Billerica, MA, USA). TNF-α, and IL-12 p40 were also measured by ELISA from R&D Systems (Burlington, ON, Canada).

### Labeling and adoptive transfer of P25TCR-Tg CD4 T cells

CD4 T cells were purified from the spleen and lymph nodes of P25TCR-Tg mice as described above. Cells were resuspended to a concentration of $10^6$ cells/mL and labeled with carboxyfluorescein succinimidyl ester from Invitrogen (Burlington, ON, Canada) at a final concentration of 5 μM. Cells were washed twice with 5% FBS/PBS to remove excess carboxyfluorescein succinimidyl ester. Approximately 2–3 × $10^6$ cells were resuspended in 200 μL of PBS and transferred into C57BL/6 mice via tail-vein injection. After 24 hrs mice were infected with *M.tb* as described.

### In vivo T cell depletion

Mice were injected i.p. with 200 μg of anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) or an IgG isotype control from Sigma-Aldrich (St. Louis, MO, USA) at day 5 postinfection. Depletion was verified using flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA).

## Statistical analysis

Asterisks in the figures indicate the level of statistical significance ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) as determined using a two-tailed Student's *t*-test or a one-way ANOVA with a Tukey post-hoc analysis. Tests were performed using GraphPad Prism software (La Jolla, CA, USA).

Acknowledgments: We would like to thank Marianne Chew for her assistance in preparing FimH, Anna Zganiacz for her assistance in preparing mycobacteria, and Xueya Feng for preparation of CD4 and CD8 T-cell depletion antibodies. This work was supported by funds to Z.X from the Canadian Institutes of Health Research (CIHR).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

## References

1 **WHO**, Global Tuberculosis Report 2012. 2012.

2 **Kendall, B. A. and Winthrop, K. L.**, Update on the epidemiology of pulmonary nontuberculous mycobacterial infections. *Semin. Respir. Crit. Care Med.* 2013. **34**: 87–94.

3 **Wolf, A. J., Linas, B., Trevejo-Nunez, G. J., Kincaid, E., Tamura, T., Takatsu, K. and Ernst, J. D.**, Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J. Immunol.* 2007. **179**: 2509–2519.

4 **Guilliams, M., Lambrecht, B. N. and Hammad, H.**, Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections. *Mucosal. Immunol.* 2013. **6**: 464–473.

5 **Chackerian, A. A., Alt, J. M., Perera, T. V., Dascher, C. C. and Behar, S. M.**, Dissemination of *Mycobacterium tuberculosis* is influenced by host factors and precedes the initiation of T-cell immunity. *Infect. Immun.* 2002. **70**: 4501–4509.

6 **Cooper, A. M. and Khader, S. A.**, The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. *Immunol. Rev.* 2008. **226**: 191–204.

7 **Cooper, A. M.**, Cell-mediated immune responses in tuberculosis. *Ann. Rev. Immunol.* 2009. **27**: 393–422.

8 **Wolf, A. J., Desvignes, L., Linas, B., Banaiee, N., Tamura, T., Takatsu, K. and Ernst, J. D.**, Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. *J. Exp. Med.* 2008. **205**: 105–115.

9 **Reiley, W. W., Shafiani, S., Wittmer, S. T., Tucker-Heard, G., Moon, J. J., Jenkins, M. K. et al.**, Distinct functions of antigen-specific CD4 T cells during murine *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 19408–19413.

10 **Jeyanathan, M., McCormick, S., Lai, R., Afkhami, S., Shaler, C. R., Horvath, C. N., Damjanovic, D. et al.**, Pulmonary *M. tuberculosis* infection delays Th1 immunity via immunoadaptor DAP12-regulated IRAK-M and IL-10 expression in antigen-presenting cells. *Mucosal. Immunol.* 2013. Doi: **10.1038/mi.2013.86**. [Epub ahead of print]

11 **Friedman, H., Yamamoto, Y. and Klein, T. W.**, Legionella pneumophila pathogenesis and immunity. *Semin. Pediatr. Infect. Dis.* 2002. **13**: 273–279.

12 **Ordway, D., Henao-Tamayo, M., Smith, E., Shanley, C., Harton, M., Troudt, J., Bai, X. et al.**, Animal model of *Mycobacterium abscessus* lung infection. *J. Leukoc. Biol.* 2008. **83**: 1502–1511.

13 **Shaler, C. R., Horvath, C., Lai, R. and Xing, Z.**, Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clin. Dev. Immunol.* 2012. **2012**: 628293.

14 **Urdahl, K. B., Shafiani, S. and Ernst, J. D.**, Initiation and regulation of T-cell responses in tuberculosis. *Mucosal. Immunol.* 2011. **4**: 288–293.

15 **Divangahi, M., Desjardins, D., Nunes-Alves, C., Remold, H. G. and Behar, S. M.**, Eicosanoid pathways regulate adaptive immunity to *Mycobacterium tuberculosis*. *Nat. Immunol.* 2010. **11**: 751–758.

16 **Blomgran, R., Desvignes, L., Briken, V. and Ernst, J. D.**, Mycobacterium tuberculosis inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host Microbe.* 2012. **11**: 81–90.

17 **Redford, P. S., Boonstra, A., Read, S., Pitt, J., Graham, C., Stavropoulos, E., Bancroft, G. J. and O'Garra, A.**, 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.* 2010. **40**: 2200–2210.

18 **Beisiegel, M., Kursar, M., Koch, M., Loddenkemper, C., Kuhlmann, S., Zedler, U., Staber, M. et al.**, Combination of host susceptibility and virulence of *Mycobacterium tuberculosis* determines dual role of nitric oxide in the protection and control of inflammation. *J. Infect. Dis.* 2009. **199**: 1222–1232.

19 **Horvath, C. N., Shaler, C. R., Jeyanathan, M., Zganiacz, A. and Xing, Z.**, Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: a critical role of airway luminal T cells. *Mucosal. Immunol.* 2012. **5**: 420–431.

20 **Fulton, S. A., Martin, T. D., Redline, R. W. and Henry Boom, W.**, Pulmonary immune responses during primary mycobacterium bovis-Calmette-Guerin bacillus infection in C57Bl/6 mice. *Am. J. Respir. Cell Mol. Biol.* 2000. **22**: 333–343.

21 **Pecora, N. D., Fulton, S. A., Reba, S. M., Drage, M. G., Simmons, D. P., Urankar-Nagy, N. J., Boom, W. H. and Harding, C. V.**, Mycobacterium bovis BCG decreases MHC-II expression in vivo on murine lung macrophages and dendritic cells during aerosol infection. *Cell Immunol.* 2009. **254**: 94–104.

22 **Fulton, S. A., Reba, S. M., Pai, R. K., Pennini, M., Torres, M., Harding, C. V. and Boom, W. H.**, Inhibition of major histocompatibility complex II expression and antigen processing in murine alveolar macrophages by *Mycobacterium bovis* BCG and the 19-kilodalton mycobacterial lipoprotein. *Infect. Immun.* 2004. **72**: 2101–2110.

23 **Rajaram, M. V., Ni, B., Morris, J. D., Brooks, M. N., Carlson, T. K., Bakthavachalu, B., Schoenberg, D. R. et al.**, Mycobacterium tuberculosis lipomannan blocks TNF biosynthesis by regulating macrophage MAPK-activated protein kinase 2 (MK2) and microRNA miR-125b. *Proc. Natl. Acad Sci. USA* 2011. **108**: 17408–17413.

24 **Balboa, L., Romero, M. M., Yokobori, N., Schierloh, P., Geffner, L., Basile, J. I., Musella, R. M. et al.**, Mycobacterium tuberculosis impairs dendritic cell response by altering CD1b, DC-SIGN and MR profile. *Immunol. Cell Biol.* 2010. **88**: 716–726.

25 **McCormick, S., Shaler, C. R. and Xing, Z.**, Pulmonary mucosal dendritic cells in T-cell activation: implications for TB therapy. *Expert. Rev. Respir. Med.* 2011. **5**: 75–85.

26 **Ballesteros-Tato, A., Leon, B., Lund, F. E. and Randall, T. D.**, Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. *Nat. Immunol.* 2010. **11**: 216–224.

27 **Steinhagen, F., Kinjo, T., Bode, C. and Klinman, D. M.**, TLR-based immune adjuvants. *Vaccine* 2011. **29**: 3341–3355.

28 **Abdul-Careem, M. F., Firoz Mian, M., Gillgrass, A. E., Chenoweth, M. J., Barra, N. G., Chan, T., Al-Garawi, A. A. et al.**, FimH, a TLR4 ligand, induces innate antiviral responses in the lung leading to protection against lethal influenza infection in mice. *Antiviral. Res.* 2011. **92**: 346–355.

29 **Khader, S. A., Partida-Sanchez, S., Bell, G., Jelley-Gibbs, D. M., Swain, S., Pearl, J. E., Ghilardi, N. et al.**, Interleukin 12p40 is required for dendritic cell migration and T cell priming after *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 2006. **203**: 1805–1815.

30 **Russell, M. S., Iskandar, M., Mykytczuk, O. L., Nash, J. H., Krishnan, L. and Sad, S.**, A reduced antigen load in vivo, rather than weak inflammation, causes a substantial delay in CD8+ T cell priming against *Mycobacterium bovis* (bacillus Calmette-Guerin). *J. Immunol.* 2007. **179**: 211–220.

31 **Divangahi, M., Behar, S. M. and Remold, H.**, Dying to live: how the death modality of the infected macrophage affects immunity to tuberculosis. *Adv. Exp. Med. Biol.* 2013. **783**: 103–120.

32 **Divangahi, M., Chen, M., Gan, H., Desjardins, D., Hickman, T. T., Lee, D. M., Fortune, S. et al.**, Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. *Nat. Immunol.* 2009. **10**: 899–906.

33 **Divangahi, M., Yang, T., Kugathasan, K., McCormick, S., Takenaka, S., Gaschler, G., Ashkar, A. et al.**, Critical negative regulation of type 1 T cell immunity and immunopathology by signaling adaptor DAP12 during intracellular infection. *J. Immunol.* 2007. **179**: 4015–4026.

34 **Myers, A. J., Marino, S., Kirschner, D. E. and Flynn, J. L.**, Inoculation dose of *Mycobacterium tuberculosis* does not influence priming of T cell responses in lymph nodes. *J. Immunol.* 2013. **190**: 4707–4716.

35 **Gonzalez-Perez, M., Marino-Ramirez, L., Parra-Lopez, C. A., Murcia, M. I., Marquina, B., Mata-Espinoza, D., Rodriguez-Miguez, Y. et al.**, Virulence and immune response induced by *Mycobacterium avium* complex strains in a model of progressive pulmonary tuberculosis and subcutaneous infection in BALB/c mice. *Infect. Immun.* 2013. **11**: 4001–4012.

36 **Arias, M. A., Jaramillo, G., Lopez, Y. P., Mejia, N., Mejia, C., Pantoja, A. E., Shattock, R. J. et al.**, Mycobacterium tuberculosis antigens specifically modulate CCR2 and MCP-1/CCL2 on lymphoid cells from human pulmonary hilar lymph nodes. *J. Immunol.* 2007. **179**: 8381–8391.

37 **Thompson, B. S., Chilton, P. M., Ward, J. R., Evans, J. T. and Mitchell, T. C.**, The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. *J. Leukoc. Biol.* 2005. **78**: 1273–1280.

38 **Baxevanis, C. N., Voutsas, I. F. and Tsitsilonis, O. E.**, Toll-like receptor agonists: current status and future perspective on their utility as adjuvants in improving anticancer vaccination strategies. *Immunotherapy* 2013. 5: 497–511.

39 **Ashkar, A. A., Mossman, K. L., Coombes, B. K., Gyles, C. L. and Mackenzie, R.**, FimH adhesin of type 1 fimbriae is a potent inducer of innate antimicrobial responses which requires TLR4 and type 1 interferon signalling. *PLoS Pathog.* 2008. 4: e1000233.

40 **Blomgran, R. and Ernst, J. D.**, Lung neutrophils facilitate activation of naive antigen-specific CD4+ T cells during *Mycobacterium tuberculosis* infection. *J. Immunol.* 2011. 186: 7110–7119.

41 **Blomgran, R., Zheng, L. and Stendahl, O.**, Uropathogenic *Escherichia coli* triggers oxygen-dependent apoptosis in human neutrophils through the cooperative effect of type 1 fimbriae and lipopolysaccharide. *Infect. Immun.* 2004. 72: 4570–4578.

42 **Leepiyasakulchai, C., Ignatowicz, L., Pawlowski, A., Kallenius, G. and Skold, M.**, Failure to recruit anti-inflammatory CD103+ dendritic cells and a diminished CD4+ Foxp3 +regulatory T cell pool in mice that display excessive lung inflammation and increased susceptibility to *Mycobacterium tuberculosis*. *Infect. Immun.* 2012. 80: 1128–1139.

43 **Santosuosso, M., Zhang, X., McCormick, S., Wang, J., Hitt, M. and Xing, Z.**, Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J. Immunol.* 2005. 174: 7986–7994.

44 **Jeyanathan, M., Damjanovic, D., Shaler, C. R., Lai, R., Wortzman, M., Yin, C., Zganiacz, A. et al.**, Differentially imprinted innate immunity by mucosal boost vaccination determines antituberculosis immune protective outcomes, independent of T-cell immunity. *Mucosal. Immunol.* 2013. 6: 612–625.

*Abbreviations:* **BAL**: bronchioalveolar lavage · **KC**: keratinocyte-derived chemokine · **dLN**: draining lymph node · **M.tb**: *Mycobacterium tuberculosis* · **NTM**: nontuberculous mycobacterial · **TB**: tuberculosis

*Full correspondence:* Dr. Zhou Xing, Department of Pathology and Molecular Medicine, and McMaster Immunology Research Centre, McMaster University, 1280 Main Street West, Room MDCL-4012, Hamilton, ON L8S 4K1, Canada
Fax: +1-905-522-6750
e-mail: xingz@mcmaster.ca

Received: 17/11/2013
Revised: 12/1/2014
Accepted: 6/2/2014
Accepted article online: 12/2/2014

# European Journal of Immunology

## Supporting Information

## for

## DOI 10.1002/eji.201344300


Rocky Lai, Mangalakumari Jeyanathan, Christopher R. Shaler, Daniela Damjanovic, Amandeep Khera, Carly Horvath, Ali A. Ashkar and Zhou Xing

**Restoration of innate immune activation accelerates Th1-cell priming and protection following pulmonary mycobacterial infection**

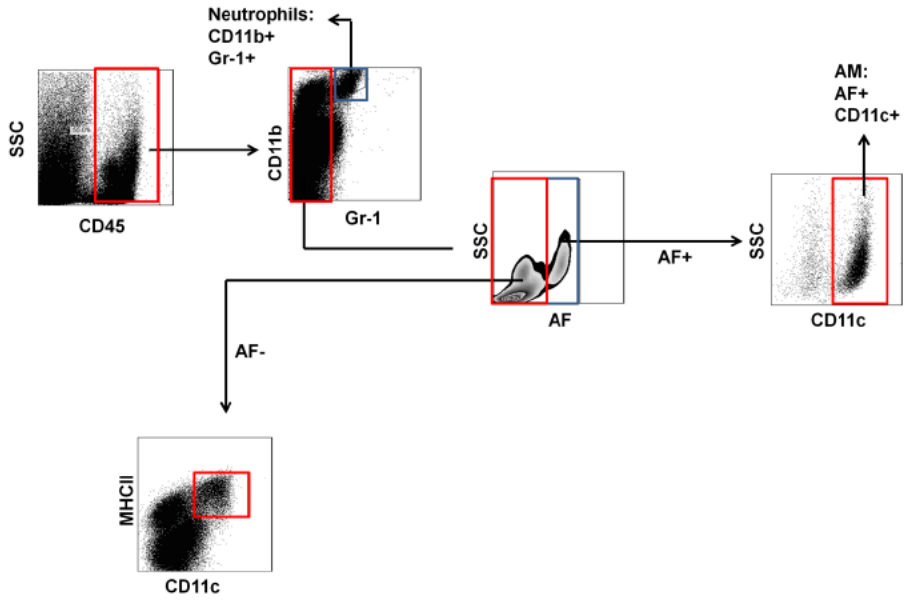



**Figure S1 – Gating strategy used to identify various APC populations within the lung**. Haematopoietic cells were identified based on their expression of CD45, after which neutrophils were excluded based on expression of Gr-1. Alveolar macrophages were separated from DCs based on their autofluorescence (AF) on the FITC channel, after which DCs were identified based on their expression of CD11c and MHCII. The data was collected using the LSRII and FACSDiva software from BD Biosciences and analyzed using FlowJo Software from Treestar.

1

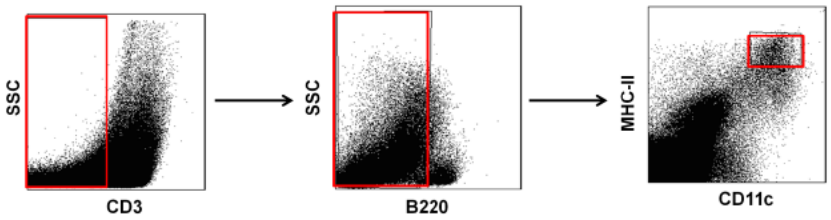



**Figure S2 –Gating strategy used to identify various APC populations within the dLN.** T cells and B cells were excluded based on their expression of CD3 and B220, respectively, after which DCs were identified based on their expression of CD11c and MHCII. Migratory DC populations are identified as being $CD11c^{high}$ $MHCII^{high}$. The data was collected using the LSRII and FACSDiva software from BD Biosciences and analyzed using FlowJo Software from Treestar.

2

# Chapter 4. What is the relative contribution of specific pulmonary DC subsets in the induction of Th1 responses following pulmonary *M.tb* infection?

**Title of Manuscript**: CD11b+ dendritic cell-mediated anti-*M.tuberculosis* Th1 activation is counter-regulated by CD103+ dendritic cells via interleukin-10

**Rocky Lai,** Mangalakumari Jeyanathan, Sam Afkhami, Anna Zganiacz, Joanne A Hammill, Yushi Yao, Charu Kaushic, Zhou Xing

In submission to *Journal of Immunology* as of September 2017

In this chapter, we seek to examine the relative importance of lung DC subsets in Th1 activation following pulmonary *M.tb* infection. Recent studies have expanded the definition of lung DC into multiple phenotypically, developmentally and functionally distinct subsets. Most prominent and well characterized of these subsets are the conventional DC (cDC) subsets, which can be distinguished based on their relative expression of CD103 and CD11b (hereby referred to as $CD103^+$cDC and $CD11b^+$cDC). While the relative ability of these two cDC subsets in $CD4^+$ and $CD8^+$ T cell activation has been addressed to some degree in various pulmonary infection models, their relative contribution to Th1 activation following pulmonary *M.tb* infection has not been well characterized. As such, we seek to elucidate their relative contribution in the induction of Th1 responses following pulmonary *M.tb* infection.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

**CD11b$^+$ dendritic cell-mediated anti-*M. tuberculosis* Th1 activation is counter-regulated by CD103$^+$ dendritic cells via interleukin-10**

Rocky Lai[1], Mangalakumari Jeyanathan[1], Sam Afkhami[1], Anna Zganiacz[1], Joanne A Hammill[1], Yushi Yao[1], Charu Kaushic[1], Zhou Xing[1*]

**Affiliations:**

[1] McMaster Immunology Research Centre, M. G. DeGroote Institute for Infectious Disease Research, and Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario, Canada.

*__Address correspondence to__: Dr. Zhou Xing, Department of Pathology and Molecular Medicine, and McMaster Immunology Research Centre, McMaster University, 1280 Main Street West, Room MDCL-4012, Hamilton, Ontario L8S 4K1, Canada.

E-mail address: xingz@mcmaster.ca

**Abstract**

*Mycobacterium tuberculosis (M.tb),* the pathogen causing pulmonary tuberculosis (TB) in humans, has evolved to delay Th1 immunity in the lung. While the conventional dendritic cells (cDC) are known to be critical to the initiation of T-cell immunity, the differential roles and molecular mechanisms of $CD11b^+$ and $CD103^+$cDC subsets in anti-*M.tb* Th1 activation remain unclear. Using a murine model of pulmonary *M.tb* infection, we found that slow arrival of *M.tb*-bearing $CD11b^+$ and $CD103^+$cDCs at the draining lymph nodes (dLN) preceded the much delayed Th1 immunity and protection in the lung. Contrary to their previously described general roles in Th polarization, $CD11b^+$cDC, but not $CD103^+$cDC, were found critically required for Th1 activation in dLN following *M.tb* infection. Rather, $CD103^+$cDC counter-regulated $CD11b^+$cDC-mediated Th1 activation directly by producing the immune-suppressive cytokine IL-10. Our study thus provides new mechanistic insights into Th immune regulation by DC subsets and helps develop novel vaccines and therapies.

(Words count: 150)

## Introduction

Pulmonary tuberculosis (TB) by *Mycobacterium tuberculosis* (*M.tb)* is the leading infectious cause of global morbidity and mortality, with an estimated 10.4 million new cases and 1.8 million deaths worldwide each year [1]. Although the current TB vaccine, BCG, has been widely used in most parts of the world for more than 6 decades, it only protects against the severe childhood forms of TB but not the highly contagious adult pulmonary TB [2]. Tremendous efforts have been made in the last few decades towards the development of novel TB vaccines to either boost or replace BCG [3-7]. However, these vaccine efforts have been hindered by the existing knowledge gap in our understanding of TB pathogenesis and protective immunity.

T lymphocytes, particularly Th1 cells, play a critical role in immunity against *M.tb* infection [8-10]. However, *M.tb* has evolved the mechanisms to suppress early immune events involved in T-cell activation, resulting in a considerable delay in the onset of Th1 immunity in the lung compared to other intracellular pathogens such as influenza virus and *Legionella pneumophila* [11-14]. Thus, following *M.tb* infection in the lung, it takes a number of days for Th1 priming to begin in the lung draining lymph nodes (dLN) [15] and the full-blown Th1 immunity does not reach to and appear in the lung until about three weeks. It is believed that this significant delay in the initiation of Th1 immunity allows *M.tb* to establish a foothold in the lung, being ineffective in clearing or controlling infection [8, 13, 14, 16]. Despite recent progress in the understanding of timing of events that leads to onset of Th1 immunity in the lung, the cellular immune mechanisms underlying the delayed Th1 responses following pulmonary *M.tb* infection still remain poorly understood.

Dendritic cells (DC) are essential in antigen presentation to prime and activate Ag-specific T-lymphocytes in dLN for the initiation of adaptive immunity [17]. There are the two main

conventional DC (cDC) subsets identified in murine lymphoid and non-lymphoid tissues, which have been differentiated based on the relative expression of their respective surface markers, CD11b and CD103 [18], with equivalent cDC subsets identified also in humans [19, 20]. Although much still remains to be unraveled, emerging evidence suggests a division of labor between $CD11b^+$cDC and $CD103^+$cDC subsets in promoting a particular type of Th polarization and responses depending on the nature of immune exposure and environmental signals [21, 22]. In general, $CD11b^+$cDC favor Th2 and Th17 immunity whereas $CD103^+$cDC are associated with Th1 and CTL responses. Specifically, $CD11b^+$cDC are involved in the initiation of Th2 activation in models of lung allergen exposure [23-25] and skin Nippostrongylus parasitic infection [26], or in the development of Th17 activation in models of lung fungal infection [27] and gut citrobacter bacterial infection [28]. On the other hand, $CD103^+$cDC are commonly linked to Th1 or type 1 immunity against intracellular parasites *Toxoplasma gondii* [29] or *Leishmania major* [30, 31] and viruses [32-34]. Furthermore, $CD103^+$cDC are also involved in facilitating the induction of regulatory T-cells [35, 36], and have recently been shown to directly inhibit Th2 allergic responses via their IL-12 production [37]. Regardless of these advances, the precise roles and molecular mechanisms of pulmonary $CD11b^+$cDC and $CD103^+$cDC subsets in Th1 activation following intracellular infection, in particular *M.tb* infection, are still largely unclear [17, 21, 38, 39]. Enhanced knowledge in this regard will be critical to unraveling the cellular and molecular mechanisms of delayed anti-TB Th1 immunity and developing novel vaccination strategies.

In the current study, using a murine model of pulmonary *M.tb* infection, we reveal that both infected $CD11b^+$cDC and $CD103^+$cDC migrate into the lung dLNs, but their migration is delayed up to 14 days after infection. Contrary to their previously understood roles in Th polarization, $CD11b^+$cDC, but not $CD103^+$cDC counterparts, are a critical player in Th1 priming

and activation following pulmonary *M.tb* exposure. Furthermore, we have identified a new role for CD103$^+$cDC and demonstrate here that CD103$^+$cDC play a direct regulatory role in anti-*M.tb* Th1 immunity by inhibiting CD11b$^+$cDC-mediated Th1 priming via the production of immune-regulatory cytokine IL-10. Our findings provide new insights into the distinct roles and functional plasticity of conventional DC subsets in the regulation of Th1 immunity against an intracellular bacterial pathogen of paramount clinical importance.

**Results**

***Ag-specific Th1 cell recruitment to the lung and bacterial control are significantly delayed following pulmonary M.tb infection***

To begin understanding the role of DC subsets in Th1 activation, we first characterized the kinetics of mycobacterial growth and T-cell responses in the lung of mice infected via pulmonary route with *M.tb*. Bacterial burden in the lung continued to increase logarithmically after infection and reached a plateau of ~6 logs by d21 post-infection (Figure S1A), in agreement with previous findings [40, 41]. Of importance, the lack of control of bacterial growth was associated with the lack of Ag-specific Th1 responses in the lung between d5 and d14 whereas the manifestation of control of bacterial growth coincided with the apparent arrival of Ag-specific Th1 cells in the lung between d14 and d21 post-infection (Figure S1B/C). These data demonstrate a close relationship between delayed Ag-specific Th1 cell immunity and uncontrolled *M.tb* replication in the lung.

***Arrival of M.tb-infected CD11b$^+$cDC and CD103$^+$cDC at the dLNs is delayed and associated with delayed Ag-specific Th1 cell priming***

In order to determine whether the delayed appearance of Ag-specific Th1 responses in the lung was due to the inability of the activated Th1 cells to enter the lung or due to delayed Th1 activation in the mediastinal lymph nodes, the lung dLN, we analyzed the kinetics of Th1 responses

in the dLN following *M.tb* infection. We found that Ag-specific Th1 activation was delayed in the dLN, and significant $CD4^+IFN\text{-}\gamma^+$T-cells were not induced until d14 post-infection (Figure 1A). Thus, marked Th1 responses at d14 in the dLN (Figure 1A) preceded the markedly delayed appearance of these cells in the lung at d21 (Figure S1B/C).

It was shown that arrival of *M.tb*-bearing DC preceded the Th1 activation in the dLN [8, 38]. Despite this, the relative migration kinetics of $CD11b^+$cDC and $CD103^+$cDC subsets have remained to be determined. As the first step towards elucidating the role of lung cDC subsets in Th1 activation, we analyzed the kinetics of major cDC subsets in the dLN at defined timepoints, d5, d10 and d14, after pulmonary *M.tb* infection. cDC subsets in the dLN were identified using a specific gating strategy as described by Ballesteros-Tato et al [42] by first excluding $CD3^+$ T-cells followed by $B220^+$ B-cells from the total live cells. From the remaining population, $CD11C^{hi}MHCII^{hi}$ cells were gated, which were further divided into $CD103^+$ and $CD103^-$ subsets. Examination of CD11b expression on these populations revealed that $CD103^+$ cells were negative for CD11b whereas $CD103^-$ cells were positive for CD11b (Figure S2). Significant increases in the number of both $CD103^+$cDC and $CD11b^+$cDC subsets were not observed in the dLN of infected mice until after d10 post-infection compared to uninfected mice (naïve) (Figure 1B). Importantly, $CD11b^+$cDC were found in significantly higher numbers than $CD103^+$cDC at d14.

Previous studies show that $CD11b^+$cDC play a role in the delivery of live *M.tb* from the lung to the dLN [38]. Since we have observed the co-migration of both $CD11b^+$ and $CD103^+$cDC subsets to the dLN, we next examined the relative bacterial load within both DC subsets to assess their antigen delivery capabilities. To this end, $CD11b^+$cDC and $CD103^+$cDC were purified from the dLN at d10 post-*M.tb* infection, and the bacterial burden within each DC subset was quantified

using a mycobacterial CFU assay. We observed that in fact, the bacterial burden within $CD103^+$cDC was significantly greater than in $CD11b^+$cDC (Figure 1C).

Together these data suggest that Th1 priming and activation is also significantly delayed in the dLN, which is associated with delayed appearance of *M.tb*-bearing $CD11b^+$ and $CD103^+$cDC in the dLN. Compared to $CD103^+$cDC, $CD11b^+$cDC are the predominant cDC subset at the time of Th1 priming (d14) in the local draining lymphoid tissue.

***$CD11b^+$cDC are superior to $CD103^+$cDC in priming and activating M.tb Ag-specific Th1 cells***

The relative role of $CD11b^+$ and $CD103^+$cDC in *M.tb*-specific Th1 priming and activation is still unclear [17, 21]. Given the close association between the arrival of *M.tb*-infected $CD11b^+$ and $CD103^+$cDC subsets at the dLN and the timing of Ag-specific Th1 priming (Figure 1A/B), we next examined the relative contribution of $CD103^+$cDC and $CD11b^+$cDC to the priming of Ag-specific Th1 cells. To address this, we purified and CFSE-labeled $CD4^+$T-cells purified from naïve P25-Tg mice engineered to express the TCR specific for an immunodominant *M.tb* antigen Ag85B [43]. CFSE-labeled P25-Tg$CD4^+$T-cells were then co-cultured with either $CD11b^+$cDC or $CD103^+$cDC purified from the dLN at d14 after *M.tb* infection at a ratio of 1:2 DC to T-cells (Figure 2A). A much greater proportion of P25-Tg$CD4^+$T-cells proliferated when co-cultured with $CD11b^+$cDC than with $CD103^+$cDC (Figure 2B -histograms) regardless of a higher infectious burden in $CD103^+$cDC (Figure 1C). This was demonstrated by significantly fewer $CD11b^+$cDC-stimulated P25-Tg$CD4^+$T-cells rested in the G0 phase and significantly more of them undergone greater-than-four rounds of proliferation (Figure 2B-bar graph). As expected, CFSE-labeled P25-Tg$CD4^+$T-cells cultured on their own did not undergo any proliferation (data not shown).

As $CD4^+$T-cell proliferative responses alone may not suffice to attest to a Th1 polarization, we measured the levels of IFN-γ, a Th1 signature cytokine, in the culture supernatants as an

indication of the ability of either CD11b$^+$cDC or CD103$^+$cDC to induce *M.tb* Ag-specific Th1 activation. In consistent with higher rates of proliferation of CD11b$^+$cDC-stimulated P25-TgCD4$^+$T-cells, these cells released much more IFN-γ than CD103$^+$cDC-stimulated counterparts (Figure 2C). Since IL-12 provides proliferative and activating signals to CD4$^+$T-cells towards Th1 polarization [44] and IL-12 production has been identified to be a primary mechanism by which CD103$^+$cDC promote Th1 activation and counter-regulate Th2 immunity [29, 30, 37], we compared IL-12 production by CD11b$^+$cDC and CD103$^+$cDC co-cultured with P25-TgCD4$^+$T-cells. Intriguingly, both cDC subsets were capable of significant and comparable levels of IL-12 production even with a higher trend seen with CD11b$^+$cDC (Figure 2D).

The above data together indicate that although both *M.tb*-infected CD11b$^+$cDC and CD103$^+$cDC migrate to the local draining lymphoid tissue following pulmonary *M.tb* infection, the former is much more potent than CD103$^+$cDC in priming and activating Ag-specific Th1 cells.

***Deficiency of CD11b$^+$cDC severely impairs Ag-specific Th1 activation following pulmonary M.tb infection***

We have thus far established that compared to CD103$^+$cDC, a greater number of infected CD11b$^+$cDC migrated to the dLN following pulmonary *M.tb* infection (Figure 1B), and CD11b$^+$cDC, on a per cell basis, were more capable of priming *M.tb* Ag-specific Th1 cells (Figure 2B/C). Therefore, we next set out to determine the role of CD11b$^+$cDC in Th1 priming *in vivo*. To this end, we made use of IRF4$^{-/-}$ mice which are largely deficient in CD11b$^+$cDC but have an intact CD103$^+$cDC population in the lung [45]. Indeed, we verified this to be the case in IRF4$^{-/-}$ mice when we examined these two cDC subsets at both d10 and d14 post-*M.tb* infection (Figure S3). In support of the major role of CD11b$^+$cDC in Th1 activation (Figure 2), we found that by d10 post-*M.tb* infection, lack of CD11b$^+$cDC led to blunted Th1 priming manifested by severely diminished

$CD4^+IFN\text{-}\gamma^+$T-cell responses in the dLN of $IRF4^{-/-}$ mice, compared to the wild type (WT) animals (Figure 3A). Impaired Ag-specific Th1 priming in the dLN of $CD11b^+$cDC-deficient animals led to severely reduced frequencies and numbers of $CD4^+IFN\text{-}\gamma^+$T-cells in the lung of these animals at d21 post-*M.tb* infection (Figure 3B) and significantly heightened bacterial loads both in the lung and spleen (Figure 3C/D).

Since besides its critical role in $CD11b^+$cDC development, IRF4 is also expressed in $CD4^+$T-cells and involved in T-cell differentiation [46], it was necessary to further verify that the diminished Th1 activation seen in *M.tb*-infected $IRF4^{-/-}$ animals was due to $CD11b^+$cDC deficiency, but not due to potential intrinsic defects in T-cells. To this end, we used a transgenic T-cell adoptive transfer approach. Thus, CFSE-labeled P25-Tg $CD4^+$T-cells purified from naïve P25-Tg mice were intravenously transferred into $IRF4^{-/-}$ or WT mice. Mice were then infected with *M.tb* one day after transfer to initiate Ag presentation by endogenous cDC. The proliferation of adoptively transferred P25-Tg$CD4^+$T-cells were assessed in the dLN at d10 post-infection. As a control, we also transferred CFSE-labeled P25-Tg$CD4^+$T-cells into animals that were not subsequently infected with *M.tb*. As expected, P25-Tg$CD4^+$T-cells transferred into uninfected control animals did not undergo proliferation (data not shown). However, up to 50% of the P25-Tg$CD4^+$T-cells transferred into infected WT $CD11b^+$cDC-competent hosts had undergone one (G1) to five (G5) rounds of proliferation (Figure 3E). In contrast, the vast majority (80%) of P25-Tg$CD4^+$T-cells transferred to infected $IRF4^{-/-}$ mice still remained in the G0 un-proliferated fraction.

These data suggest that $CD11b^+$cDC play a critical role in Th1 priming and activation in lung draining lymphoid tissue and subsequent Th1 immunity in the lung following pulmonary *M.tb* infection.

***CD11b$^+$cDC-mediated M.tb Ag-specific Th1 priming and activation is counter-regulated by CD103$^+$cDC***

Although we have found a key role for CD11b$^+$cDC in Th1 priming and activation following pulmonary *M.tb* infection, both CD11b$^+$cDC and CD103$^+$cDC subsets were infected by *M.tb* (Figure 1C) and co-migrated to the dLN (Figure 1B), all of which coincided with Th1 priming in the dLN (Figure 1A). These lines of evidence suggest that the pace and intensity of Th1 priming in the local lymphoid tissue may be determined by the interaction between these two cDC subsets. To address this question, we adopted an *ex vivo* co-culture system. To this end, CFSE-labeled P25-TgCD4$^+$T-cells purified from naïve animals were co-cultured either with CD11b$^+$cDC alone or together with CD103$^+$cDC in a 1:1 ratio. The cDC subsets were purified from the dLN of WT mice at d14 post-pulmonary *M.tb* infection. Although we observed that P25-TgCD4$^+$T-cells proliferated under both conditions, contrary to our initial postulate, the interaction of CD11b$^+$cDC with CD103$^+$cDC in co-culture led to markedly reduced proliferative rates of P25-TgCD4$^+$T-cells, resulting in significantly increased T-cells in the resting phase (G0) (Figure 4A). Such markedly reduced T-cell proliferation seen with CD11b$^+$cDC co-cultured with CD103$^+$cDC was accompanied by significantly reduced IFN-γ levels in co-culture supernatants (Figure 4B). Since we and others have found *M.tb*-infected Ag-presenting cells to be also capable of IFN-γ production [47, 48], to confirm that reduced IFN-γ levels in co-cultures resulted directly from suppressed Th1 activation, but not from altered DC function, we quantified CD4$^+$IFN-γ$^+$T-cells by intracellular cytokine staining and flow cytometry. In line with reduced IFN-γ levels in the culture supernatants, the frequency of CD4$^+$IFN-γ$^+$T-cells was significantly reduced by 50% when CD11b$^+$cDC were co-cultured with CD103$^+$cDC (Figure 4C). Since we have seen comparably significant production of IL-12 by either CD11b$^+$cDC or CD103$^+$cDC when cultured alone with CD4$^+$T-cells (Figure

2D), we assessed and compared the net IL-12 production by CD11b$^+$cDC with that from co-cultures of CD11b$^+$cDC and CD103$^+$cDC. Surprisingly, there were remarkably reduced IL-12 levels when CD11b$^+$cDC were co-cultured with CD103$^+$cDC (Figure 4D). These data, at least in an *ex vivo* setting, suggest a negative regulatory role of CD103$^+$cDC in CD11b$^+$cDC-mediated *M.tb* Ag-specific Th1 activation.

To further investigate the immune regulatory relationship between CD11b$^+$cDC and CD103$^+$cDC in an *in vivo* setting, mice were challenged with *M.tb*, and CD11b$^+$cDC and CD103$^+$cDC were purified from the dLN of infected animals at d14 (Figure 5A). Purified CD11b$^+$cDC, CD103$^+$cDC, or mixed CD11b$^+$cDC and CD103$^+$cDC (1:1) preparations were adoptively transferred into naïve CD45.1 congenic animals that were already engrafted with *M.tb* Ag-specific P25-TgCD4$^+$T-cells. The levels of CD45.2$^+$P25-TgCD4$^+$T-cell responses to *M.tb* Ag-bearing cDCs were assessed as the total numbers of adoptively transferred CD45.2$^+$CD4$^+$T-cells in the dLN at d3 and d7 post-cDC transfer (Figure 5A). At d3, very small numbers of *M.tb* Ag-specific CD45.2$^+$CD4$^+$T-cells were detected in the lung dLN under all three conditions, although there was a higher trend of T-cell responses in the animals received CD11b$^+$cDC alone (Figure 5B). However, by d7 while P25-TgCD4$^+$T-cell numbers increased in both CD11b$^+$cDC and CD103$^+$cDC alone groups compared to d3, CD11b$^+$cDC led to a much greater increase in such T-cells than CD103$^+$ counterparts (Figure 5B), consistent with a greater capacity of CD11b+cDC to drive Th1 activation seen in our *ex vivo* model system (Figure 2). Of importance, in the mice that received both CD103$^+$cDC and CD11b$^+$cDC, CD103$^+$cDC significantly suppressed CD11b$^+$cDC-induced Ag-specific CD45.2$^+$CD4$^+$T-cell responses (Figure 5B).

Taken together, these data demonstrate that CD11b$^+$cDC play a dominant role in Th1 priming and activation in the lung draining lymphoid tissue following pulmonary *M.tb* infection and CD103$^+$cDC act to negatively regulate CD11b$^+$cDC-mediated Th1 activation.

***CD103$^+$cDC, but not CD11b$^+$cDC, is a significant source of IL-10 following pulmonary M.tb infection***

Having established that the crosstalk between CD11b$^+$cDC and CD103$^+$cDC impacts the level of Th1 activation in dLN, we next sought to determine the molecular mechanisms. We decided to first focus on examining IL-12 and IL-10 production as the former is a critical Th1-polarizing cytokine, whereas the latter is a potent immune regulatory molecule in anti-TB Th1 immunity [44, 49]. To this end, CD11b$^+$cDC and CD103$^+$cDC were purified from dLN of animals at d14 post-*M.tb* infection, *ex vivo* cultured individually, and stimulated with *M.tb* antigens. In basic agreement with the data in Figure 2D, both CD11b$^+$cDC and CD103$^+$cDC subsets, when cultured alone without T-cells, produced comparable amounts of IL-12 (Figure 6A). However, CD11b$^+$cDC alone had a poor ability to produce the immune regulatory cytokine IL-10, in sharp contrast to significant IL-10 production by CD103$^+$cDC (Figure 6A).

To further investigate IL-10 production by the cDC subsets, we adopted a cDC-T-cell co-cultured system described for Figure 2 where CD11b$^+$cDC and CD103$^+$cDC were purified from the dLN of d14 *M.tb*-infected mice and each was co-cultured with *M.tb* Ag-specific P25-Tg CD4$^+$T-cells purified from naïve P25-Tg mice. IL-10 protein levels in the culture supernatants were quantified by ELISA. Indeed, significantly greater amounts of IL-10 were measured in the cultures of CD103$^+$cDC and P25-TgCD4$^+$T-cells than in CD11b$^+$cDC co-cultures (Figure 6B). To determine the relative contribution of both cDC and CD4$^+$T-cells to IL-10 production measured in the co-culture system, we performed intracellular IL-10 cytokine staining and flow cytometry

analysis. We found that in accordance with heightened IL-10 production measured in culture supernatants (Figure 6B), there were high and significantly greater frequencies of $CD103^+$cDC producing IL-10 than $CD11b^+$cDC counterparts (Figure 6C –histogram & bar graph). By comparison, the frequencies of P25-Tg$CD4^+$T-cells positive for IL-10 were not only tiny but were also very similar when cultured with either cDC subset (Figure S4),

The above data together indicate that while both $CD11b^+$cDC and $CD103^+$cDC can produce Th1-polarizing cytokine IL-12, $CD103^+$cDC represents the major source of immune suppressive cytokine IL-10.

***$CD11b^+$cDC-mediated anti-M.tb Th1 activation is counter-regulated by $CD103^+$cDC via IL-10 production***

Having demonstrated that $CD11b^+$cDC is a robust driver of anti-*M.tb* Th1 activation which is subject to inhibition imposed by $CD103^+$cDC (Figures 2-5), and that the latter is a major source of IL-10 (Figure 6), we next set out to determine the role of IL-10 produced by $CD103^+$cDC in $CD11b^+$cDC-mediated Th1 activation. We first examined if exogenously introduced IL-10 protein would inhibit $CD11b^+$cDC-mediated *M.tb* Ag-specific Th1 activation. To this end, $CD11b^+$cDC were isolated from the dLN of *M.tb*-infected animals and co-cultured with CFSE-labelled P25-Tg$CD4^+$T-cells either in the absence or presence of recombinant murine IL-10 protein (rIL-10). Indeed, introduction of rIL-10 inhibited the extent of $CD11b^+$cDC-stimulated $CD4^+$T-cells proliferation as indicated by significantly increased frequencies of cells in G3 and G4 but significantly decreased frequencies of the cells past G5 (Figure 7A). Furthermore, the presence of rIL-10 resulted in markedly reduced production of Th1 cytokine IFN-γ by $CD4^+$T-cells (Figure 7B), which was concurrently accompanied by markedly decreased production of Th1-polarizing cytokine IL-12 from $CD11b^+$cDC (Figure 7C).

To further examine the role of IL-10 in the inhibition of CD11b$^{+}$cDC-mediated Th1 activation, CD11b$^{+}$cDC and CD103$^{+}$cDC were isolated from the dLN of *M.tb*-infected animals, mixed at a 1:1 ratio, and co-cultured with CFSE-labelled P25-TgCD4$^{+}$T-cells. IL-10 signalling in co-cultures was blocked by using an anti-IL-10 receptor mAb (anti-IL10R). IL-10R blockade led to increased proliferation of P25-TgCD4$^{+}$T-cells as indicated by significantly decreased frequencies of cells in G1 to G4 phases but significantly increased frequencies of the cells past the G5 phase (Figure 7D). Of importance, besides increased CD4$^{+}$T-cell proliferation, IL-10R blockade resulted in markedly increased production of Th1 cytokine IFN-γ by T-cells (Figure 7E). In consistent with the earlier data (Figure 4D), co-culture of CD11b+cDC with CD103+cDC led to markedly reduced production of IL-12 (Figure 7F), which was restored with IL-10R blockade (Figure 7F).

Together these data suggest that CD103$^{+}$cDC is a negative regulator of Th1 immunity and it inhibits CD11b$^{+}$cDC-mediated anti-*M.tb* Th1 priming and activation via the action of immune suppressive IL-10.

**Discussion**

Our enhanced understanding into the cellular and molecular mechanisms underlying the delayed onset of Th1 immunity following pulmonary *M.tb* infection is critical to the development of effective prophylactics and therapeutics against TB. Although the host is able to mount a robust Th1 response to *M.tb*, the delay in the initiation of this response impedes effective control of the infection [8, 13, 14, 16] (Figure S5). Given that the initiation of Th1 immunity is dependent on innate immune activation, it is important to understand how the early innate immune events are impacted by *M.tb* infection in order to design the immune strategies for accelerating Th1 immunity in the lung [41, 50].

DC are critical to the initiation of Th1 cell responses as they migrate to the lung dLN to prime Ag-specific T-cells following pulmonary *M.tb* infection [38, 51], and thus may represent a critical checkpoint that *M.tb* manipulates to counter host defense. Recent progress has identified the heterogeneity within the previously defined $CD11c^{+}MHC\text{-}II^{+}$ conventional DCs (cDC) consisting of $CD11b^{+}$cDC and $CD103^{+}$cDC [17, 21, 22]. These cDC subsets have recently been shown to play non-redundant roles in orchestrating specific Th activation in different immune settings [19, 23, 24, 27, 29, 31, 32]. However, the roles of both DC subsets in anti-TB Th1 activation remain poorly defined. Here we show a functional dichotomy between $CD11b^{+}$cDC and $CD103^{+}$cDC after pulmonary *M.tb* infection. We find that it takes 10-14 days for these cDC subsets to migrate to the lung dLN, which leads to subsequently delayed Th1 priming and Th1 immunity in the dLN and lung following pulmonary *M.tb* infection (Figure S5). Contrary to their respectively reported Th polarizing preferences, we find that it is the $CD11b^{+}$cDC, but not the $CD103^{+}$cDC counterpart, that is a major driver of *M.tb* Ag-specific Th1 activation. Rather, we provide strong evidence that $CD103^{+}$cDC play a direct immune-suppressive role in $CD11b^{+}$cDC-mediated Th1 priming via IL-10 production. Our study has thus identified a novel immune suppressive mechanism mediated by a specific cDC subset and operative in the dLN that contributes to the overall delayed Th1 immunity in the lung, following pulmonary *M.tb* infection.

The cDC are adept at activating naïve T-cells and initiating adaptive immunity. Recent studies have described varied roles for cDC subsets in T-cell polarization depending on the immunologic condition. In this regard, although previous studies have reported a role for $CD11c^{+}CD11b^{+}$ DC in delivering viable *M.tb* to the dLN [38], these studies have not studied the specific cDC subsets involved. Here by using a more comprehensive set of cDC markers [42] we have identified the two major *M.tb*-bearing cDC subsets, $CD11b^{+}$cDC and $CD103^{+}$cDC, to migrate

to the lung dLNs following *M.tb* infection. However, we find the CD103$^+$cDC to be infected by *M.tb* to a greater extent than CD11b$^+$cDC. A similar increased susceptibility was observed in CD103$^+$cDC in a model of influenza infection [52]. The heightened *M.tb* load in CD103$^+$cDC can be ascribed to their anatomic location within the epithelial layer [17] (Figure S5), providing them with easier access to sample the bacteria entering the airway. Up to date, CD11b$^+$cDC have been found to be critical primarily to Th2 and Th17 activation in the models of allergy and extracellular parasitic or fungal infection [23-28], whereas CD103$^+$cDC mainly initiate anti-intracellular parasitic Th1 activation [29-31] or anti-viral type 1 CD8$^+$ effector T-cell responses [32, 53]. Our study provides novel evidence that CD11b$^+$cDC are the principal cDC to initiate Th1 responses to intracellular *M.tb* infection (Figure S5), contrasting with a rather weak ability to do so by CD103$^+$cDC. Our findings suggest that the particular division of labor and relative roles in Th polarization between these two important cDC subsets are highly contextual and cannot be generalized.

In our study, the critical role of CD11b$^+$cDC in *M.tb*-specific Th1 activation is further supported by the findings from IRF4-deficient animals. As a result of CD11b$^+$cDC deficiency, these animals demonstrated blunted Ag-specific Th1 responses and immune protection following pulmonary *M.tb* infection. The lack of Th1 priming in IRF4$^{-/-}$ mice is likely not due to an inherent inability of these mice to generate a Th1 response as CD4$^+$T-cells from IRF4$^{-/-}$ mice have previously been shown to produce IFN-γ under Th1-polarizing conditions [54-56]. As shown in our current study and by others [45, 57], IRF4$^{-/-}$ mice have intact CD103$^+$cDC and CD8$^+$ residential DC. Our data further suggests that in the absence of CD11b$^+$cDC, CD103$^+$cDC alone are not sufficient in rescuing impaired Th1 responses following *M.tb* infection.

Our study also reveals a newly identified function of CD103$^+$cDC. Rather than being a critical driver of Th1 polarization, CD103$^+$cDC play an immune suppressive role via their IL-10

production in the process of CD11b$^+$cDC-driven Th1 activation in the dLN following *M.tb* infection (Figure S5). CD103$^+$cDC have recently been demonstrated to directly counter-regulate allergic Th2 immunity via their IL-12 production [37]. Some other immune regulatory activities of CD103$^+$cDC are ascribed indirectly to their induction of $T_{reg}$ cells [35, 36, 58]. Our study reveals a different direct immune regulatory role by CD103$^+$cDC via their IL-10 production following pulmonary *M.tb* infection. Our study has thus identified a novel cellular and molecular mechanism operative in the lung draining lymphoid tissue upstream of the recruitment of anti-*M.tb* Th1 cells to the lung. Thus, both delayed DC migration from the lung to the dLN and suppressed Th1 priming in the dLN contribute to the overall retarded onset of Th1 immunity in the lung. While IL-10 has been implicated in the suppression of Th1 responses to *M.tb* infection [49, 59, 60] and pulmonary DC was shown capable of IL-10 production [61, 62], the cellular source of IL-10 and its functionality within the dLN has never been defined. Our study provides a clear link between Th1 suppressive activity of CD103$^+$cDC and IL-10 production. Our data generated by using rIL-10 or IL-10R-blocking mAb further suggest that CD103$^+$cDC-derived IL-10 acts on CD11b$^+$cDC and Th cells to suppress Th1 activation (Figure S5).

In summary, we have found that it takes many days before both *M.tb*-bearing CD11b$^+$ and CD103$^+$cDC subsets co-arrive at the dLN, which precedes much delayed protective Th1 immunity in the lung following *M.tb* infection. CD11b$^+$cDC, but not CD103$^+$cDC, are essential to Th1 cell priming. CD103$^+$cDC, however, dampens CD11b$^+$cDC-induced Th1 activation via IL-10 production in the dLN. Our study thus provides novel mechanistic insights into the distinct roles of cDC subsets in the regulation of Th1 immunity following an intracellular bacterial infection of clinical importance and holds implications in the development of effective vaccine and therapeutic strategies.

## Materials and Methods

### Mice

Female C57BL/6 mice (6-8 weeks old) were purchased from Charles River (Wilmington, MA, USA). P25-Tg mice (C57BL/6-Tg(H2-Kb-Tcra,-Tcrb)P25Ktk/J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). IRF4$^{-/-}$ breeding mice were kindly provided by Dr. Tak Mak (Princess Margaret Cancer Centre, University Health Network, Toronto, Canada) and bred in-house within the animal facility at McMaster University. All experimental mice were housed within the Level 3 Biosafety Facility at McMaster University. All experiments were conducted in accordance with the McMaster University Animal Research Ethics Board.

### *Mycobacterium tuberculosis* infection

The *M.tb*H37Rv strain was prepared for infection and delivered intranasally as previously described at a dose of 1x10$^4$ bacteria[41, 63]. Bacterial burden was assessed at each experimental timepoint by plating serial dilutions of lung or spleen homogenates in triplicates onto Middlebrook 7H10 plates and incubated at 37$^o$C for 15-17 days before enumeration.

### Mononuclear cell isolation and DC purification

Mononuclear cells were isolated from the lungs, spleen and lymph nodes of *M.tb* infected mice at the indicated timepoints as described [41, 63] (see Supplemental methods for isolation of cells from the lymph node). All cells were resuspended in RPMI supplemented with 10% FBS, 1% Pen-Strep and 1% L-Glutamine (cRPMI) and counted using Scepter cell counter from Millipore (Etobicoke, ON, CA). Dendritic cells were further purified from mononuclear cells isolated from the lung dLN 14 days post infection using a multi-sort approach (see Supplemental methods).

**Cell surface and intracellular cytokine immunostaining**

Mononuclear cells isolated from the lung or lymph nodes and cultured in a 96 well plate at a concentration of $20x10^6$ cells/mL stimulated with crude BCG and *M.tb* culture filtrate proteins for 24 hours as described [41, 64]. The following antibodies were used for identifying immune cells and cellular responses: CD3-V450, CD4-PE-Cy7, CD8a-APC-Cy7, IFN-γ-APC, IL-10-APC, CD45-APC-Cy7, CD11b-PE-Cy7 CD11b-BV711, CD11c-APC from BD Biosciences (San Jose, CA, USA); MHC-II (I-A/I-E)-Alexa Fluor 700 and CD103-Biotin from eBiosciences (San Diego, CA, USA); Ly6C/G-Pacific Orange and Qdot800 from Invitrogen (Burlington, ON, Canada), CD11b-BV711 from Biolegend (San Diego, CA, USA). All flow cytometry data was collected using the LSRII and FACSDiva software from BD Biosciences (San Jose, CA, USA) and analyzed using FlowJo Software from Treestar (Ashland, OR, USA).

**Transgenic T-cell purification, CFSE labelling, and *in vivo* and *ex vivo* antigen presentation assays**

$CD4^+$T-cells were further purified from the mononuclear cells isolated from the spleen and lymph nodes of P25-Tg mice using a Mouse $CD4^+$T-cell negative selection kit according to manufacturer's instructions (Stemcell Technologies, Vancouver, CA). Purity (>90%) was verified by flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA). $CD4^+$T-cells were labelled with Carboxyfluorescein succinimidyl ester (CFSE) from Invitrogen (Burlington, ON, Canada) at a final concentration of 5μM and either transferred i.v. into recipient mice or used in our antigen presentation assay (see Supplemental methods)

## Statistical analysis

Asterisks in the figures indicate the level of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)) as determined using a two-tailed student's T-test, One-Way or Two-way ANOVA with a Tukey post-hoc analysis. Tests were performed using GraphPad Prism software (La Jolla, CA, USA). Data are expressed as mean±SD unless otherwise stated.

## Acknowledgements

This study was supported by funds from the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council of Canada, and the Canadian Foundation for Innovation. We are grateful to the provision of IRF4KO mouse breeders by Dr. Tak Mak and to the technical assistance by the BSL3 facility animal care staff.

## Author contributions

R.L., M.J. and Z.X. conceived and designed the study. S.A., A.Z., J.A.H. and Y.Y performed the experiments. R.L. and M.J. performed data analysis. C.K. provided critical reagents. R.L. M.J. and Z.X. wrote the manuscript.

## Conflict of interest

The authors declare no conflicts of interest.

## References


1. WHO. Global Tuberculosis Report 2016. 2016.

2. Brennan MJ, Thole J. Tuberculosis vaccines: a strategic blueprint for the next decade. *Tuberculosis (Edinb)* 2012; **92 Suppl 1:** S6-13.

3. Xing Z, Jeyanathan M, Smaill F. New approaches to TB vaccination. *Chest* 2014; **146**(3)**:** 804-812.

4. Kaufmann SH. Tuberculosis vaccines: time to think about the next generation. *Semin Immunol* 2013; **25**(2)**:** 172-181.

5. Ottenhoff TH, Kaufmann SH. Vaccines against tuberculosis: where are we and where do we need to go? *PLoS Pathog* 2012; **8**(5)**:** e1002607.

6. Meyer J, McShane H. The next 10 years for tuberculosis vaccines: do we have the right plans in place? *Expert Rev Vaccines* 2013; **12**(4)**:** 443-451.

7. Andersen P, Kaufmann SH. Novel vaccination strategies against tuberculosis. *Cold Spring Harb Perspect Med* 2014; **4**(6).

8. Cooper AM. Cell-Mediated Immune Responses in Tuberculosis. *Annual Review of Immunology* 2009; **27**(1)**:** 393-422.

9. Philips JA, Ernst JD. Tuberculosis pathogenesis and immunity. *Annu Rev Pathol* 2012; **7:** 353-384.

10. Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM. In search of a new paradigm for protective immunity to TB. *Nat Rev Microbiol* 2014; **12**(4)**:** 289-299.

11. Friedman H, Yamamoto Y, Klein TW. Legionella pneumophila pathogenesis and immunity. *Semin Pediatr Infect Dis* 2002; **13**(4)**:** 273-279.

12. La Gruta NL, Turner SJ. T cell mediated immunity to influenza: mechanisms of viral control. *Trends Immunol* 2014; **35**(8)**:** 396-402.

13. Shaler CR, Horvath C, Lai R, Xing Z. Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clin Dev Immunol* 2012; **2012:** 628293.

14. Urdahl KB. Understanding and overcoming the barriers to T cell-mediated immunity against tuberculosis. *Semin Immunol* 2014; **26**(6)**:** 578-587.

15. Chackerian AA, Alt JM, Perera TV, Dascher CC, Behar SM. Dissemination of Mycobacterium tuberculosis is influenced by host factors and precedes the initiation of T-cell immunity. *Infect Immun* 2002; **70**(8)**:** 4501-4509.

16. Urdahl KB, Shafiani S, Ernst JD. Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol* 2011; **4**(3)**:** 288-293.

17. Guilliams M, Lambrecht BN, Hammad H. Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections. *Mucosal Immunol* 2013; **6**(3)**:** 464-473.

18. Satpathy AT, Wu X, Albring JC, Murphy KM. Re(de)fining the dendritic cell lineage. *Nat Immunol* 2012; **13**(12)**:** 1145-1154.

19. Murphy TL, Grajales-Reyes GE, Wu X, Tussiwand R, Briseno CG, Iwata A *et al*. Transcriptional Control of Dendritic Cell Development. *Annu Rev Immunol* 2016; **34:** 93-119.

20. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 2013; **31:** 563-604.

21. Kopf M, Schneider C, Nobs SP. The development and function of lung-resident macrophages and dendritic cells. *Nat Immunol* 2015; **16**(1)**:** 36-44.

22. Cook PC, MacDonald AS. Dendritic cells in lung immunopathology. *Semin Immunopathol* 2016; **38**(4)**:** 449-460.

23. Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W *et al.* Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013; **38**(2)**:** 322-335.

24. Williams JW, Tjota MY, Clay BS, Vander Lugt B, Bandukwala HS, Hrusch CL *et al.* Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nature communications* 2013; **4:** 2990.

25. Medoff BD, Seung E, Hong S, Thomas SY, Sandall BP, Duffield JS *et al.* CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J Immunol* 2009; **182**(1)**:** 623-635.

26. Gao Y, Nish SA, Jiang R, Hou L, Licona-Limon P, Weinstein JS *et al.* Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* 2013; **39**(4)**:** 722-732.

27. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D *et al.* IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* 2013; **38**(5)**:** 970-983.

28. Satpathy AT, Briseno CG, Lee JS, Ng D, Manieri NA, Kc W *et al.* Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat Immunol* 2013; **14**(9)**:** 937-948.

29. Mashayekhi M, Sandau MM, Dunay IR, Frickel EM, Khan A, Goldszmid RS *et al.* CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. *Immunity* 2011; **35**(2)**:** 249-259.

30. Martinez-Lopez M, Iborra S, Conde-Garrosa R, Sancho D. Batf3-dependent CD103+ dendritic cells are major producers of IL-12 that drive local Th1 immunity against Leishmania major infection in mice. *Eur J Immunol* 2015; **45**(1)**:** 119-129.

31. Ashok D, Schuster S, Ronet C, Rosa M, Mack V, Lavanchy C *et al.* Cross-presenting dendritic cells are required for control of Leishmania major infection. *Eur J Immunol* 2014; **44**(5)**:** 1422-1432.

32. Desch AN, Randolph GJ, Murphy K, Gautier EL, Kedl RM, Lahoud MH *et al.* CD103+ pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *J Exp Med* 2011; **208**(9)**:** 1789-1797.

33. GeurtsvanKessel CH, Willart MA, van Rijt LS, Muskens F, Kool M, Baas C *et al.* Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J Exp Med* 2008; **205**(7)**:** 1621-1634.

34. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M *et al.* Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 2008; **322**(5904)**:** 1097-1100.

35. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y *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* 2007; **204**(8)**:** 1757-1764.

36. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR *et al.* Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 2007; **204**(8)**:** 1775-1785.

37. Everts B, Tussiwand R, Dreesen L, Fairfax KC, Huang SC, Smith AM *et al.* Migratory CD103+ dendritic cells suppress helminth-driven type 2 immunity through constitutive expression of IL-12. *J Exp Med* 2016; **213**(1)**:** 35-51.

38. Wolf AJ, Linas B, Trevejo-Nunez GJ, Kincaid E, Tamura T, Takatsu K *et al.* Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J Immunol* 2007; **179**(4)**:** 2509-2519.

39. Reljic R, Di Sano C, Crawford C, Dieli F, Challacombe S, Ivanyi J. Time course of mycobacterial infection of dendritic cells in the lungs of intranasally infected mice. *Tuberculosis (Edinb)* 2005; **85**(1-2)**:** 81-88.

40. Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, Takatsu K *et al.* Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J Exp Med* 2008; **205**(1)**:** 105-115.

41. Lai R, Jeyanathan M, Shaler CR, Damjanovic D, Khera A, Horvath C *et al.* Restoration of innate immune activation accelerates Th1 cell priming and protection following pulmonary mycobacterial infection. *Eur J Immunol* 2014.

42. Ballesteros-Tato A, Leon B, Lund FE, Randall TD. Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. *Nat Immunol* 2010; **11**(3)**:** 216-224.

43. Tamura T, Ariga H, Kinashi T, Uehara S, Kikuchi T, Nakada M *et al.* The role of antigenic peptide in CD4+ T helper phenotype development in a T cell receptor transgenic model. *Int Immunol* 2004; **16**(12)**:** 1691-1699.

44. Cooper AM, Khader SA. The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. *Immunol Rev* 2008; **226:** 191-204.

45. Bajana S, Turner S, Paul J, Ainsua-Enrich E, Kovats S. IRF4 and IRF8 Act in CD11c+ Cells To Regulate Terminal Differentiation of Lung Tissue Dendritic Cells. *J Immunol* 2016; **196**(4)**:** 1666-1677.

46. Huber M, Lohoff M. IRF4 at the crossroads of effector T-cell fate decision. *Eur J Immunol* 2014; **44**(7)**:** 1886-1895.

47. Wang J, Wakeham J, Harkness R, Xing Z. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest* 1999; **103**(7)**:** 1023-1029.

48. Fricke I, Mitchell D, Mittelstadt J, Lehan N, Heine H, Goldmann T *et al.* Mycobacteria induce IFN-gamma production in human dendritic cells via triggering of TLR2. *J Immunol* 2006; **176**(9)**:** 5173-5182.

49. Redford PS, Murray PJ, O'Garra A. The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunol* 2011; **4**(3)**:** 261-270.

50. Griffiths KL, Ahmed M, Das S, Gopal R, Horne W, Connell TD *et al.* Targeting dendritic cells to accelerate T-cell activation overcomes a bottleneck in tuberculosis vaccine efficacy. *Nature communications* 2016; **7:** 13894.

51. Khader SA, Partida-Sanchez S, Bell G, Jelley-Gibbs DM, Swain S, Pearl JE *et al.* Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J Exp Med* 2006; **203**(7)**:** 1805-1815.

52. Hao X, Kim TS, Braciale TJ. Differential response of respiratory dendritic cell subsets to influenza virus infection. *J Virol* 2008; **82**(10)**:** 4908-4919.

53. del Rio ML, Rodriguez-Barbosa JI, Kremmer E, Forster R. CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J Immunol* 2007; **178**(11)**:** 6861-6866.

54. Murphy TL, Tussiwand R, Murphy KM. Specificity through cooperation: BATF-IRF interactions control immune-regulatory networks. *Nat Rev Immunol* 2013; **13**(7)**:** 499-509.

55. Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH. Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J Exp Med* 2002; **195**(8)**:** 1003-1012.

56. Tominaga N, Ohkusu-Tsukada K, Udono H, Abe R, Matsuyama T, Yui K. Development of Th1 and not Th2 immune responses in mice lacking IFN-regulatory factor-4. *Int Immunol* 2003; **15**(1)**:** 1-10.

57. Tamura T, Tailor P, Yamaoka K, Kong HJ, Tsujimura H, O'Shea JJ *et al.* IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol* 2005; **174**(5)**:** 2573-2581.

58. Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL *et al.* Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med* 2008; **205**(9)**:** 2139-2149.

59. Demangel C, Bertolino P, Britton WJ. 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* 2002; **32**(4)**:** 994-1002.

60. Jeyanathan M, McCormick S, Lai R, Afkhami S, Shaler CR, Horvath CN *et al.* Pulmonary M. tuberculosis infection delays Th1 immunity via immunoadaptor DAP12-regulated IRAK-M and IL-10 expression in antigen-presenting cells. *Mucosal Immunol* 2013.

61. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001; **2**(8)**:** 725-731.

62. Kushwah R, Hu J. Role of dendritic cells in the induction of regulatory T cells. *Cell Biosci* 2011; **1**(1)**:** 20.

63. Jeyanathan M, Thanthrige-Don N, Afkhami S, Lai R, Damjanovic D, Zganiacz A *et al.* Novel chimpanzee adenovirus-vectored respiratory mucosal tuberculosis vaccine: overcoming local anti-human adenovirus immunity for potent TB protection. *Mucosal Immunol* 2015; **8**(6)**:** 1373-1387.

64. Jeyanathan M, Damjanovic D, Shaler CR, Lai R, Wortzman M, Yin C *et al.* Differentially imprinted innate immunity by mucosal boost vaccination determines antituberculosis immune protective outcomes, independent of T-cell immunity. *Mucosal Immunol* 2013; **6**(3)**:** 612-625.

**Figure legends**

**Figure 1. Delayed Ag-specific Th1 cell priming in the dLN associates with delayed appearance of *M.tb* infected CD11b$^+$cDC and CD103$^+$cDC in the dLN.** (A) Representative dotplots and bar graph showing frequencies and numbers of Ag-specific CD4$^+$IFN-γ$^+$ T-cells, respectively, in the dLN at designated timepoints following *M.tb* infection. (B) Representative zebraplots and bar graph showing frequencies and numbers of CD103$^+$ and CD11b$^+$cDC, respectively, in the dLN. (C) Bar graph comparing *M.tb* burden in CD103$^+$ and CD11b$^+$cDC purified from dLN after *M.tb* infection. Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

**Figure 2. CD11b$^+$cDC potently activate *M.tb* Ag-specific Th1 responses compared to CD103$^+$cDC.** (A) Experimental schema. (B) Representative histogram showing degrees of proliferation of P25-Tg CD4$^+$ T-cells when co-cultured with either CD103$^+$ or CD11b$^+$cDC. Bar graph comparing frequencies of P25-Tg CD4$^+$ T-cells in each generation when co-cultured with either CD103$^+$ or CD11b$^+$cDC. Bar graphs showing levels of IFN-γ (C) and IL-12 (D) in the supernatants of the co-cultures measured by ELISA. Data are expressed as mean±SD of samples plated in triplicates, representative of 3 independent experiments.

**Figure 3. Deficiency of CD11b$^+$cDC severely impairs *M.tb* Ag-specific Th1 activation and immune protection.** Representative dotplots and bar graphs showing frequencies and numbers of Ag-specific CD4$^+$IFN-γ$^+$ T-cells, respectively, in the dLN (A) and the lung (B) of wild type (WT) and CD11b$^+$cDC deficient (IRF4$^{-/-}$) mice following pulmonary *M.tb* infection. (C) & (D) Bar graphs comparing *M.tb* burden in the lung and spleen, respectively, of WT and IRF4$^{-/-}$ mice following *M.tb* infection. (E) Representative histograms showing degrees of proliferation of P25-Tg CD4$^+$ T-cells adoptively transferred to WT and IRF4$^{-/-}$ mice. Bar graph comparing frequencies

of P25-Tg $CD4^+$ T-cells in the resting state (G0) and proliferated up to $5^{th}$ generation (G1-G5) in WT and $IRF4^{-/-}$ mice. Data are expressed as mean±SD of three mice (A/B/E) or five mice (C/D)/group, representative of 2 independent experiments.

**Figure 4. $CD11b^+$cDC-mediated *M.tb* Ag-specific Th1 priming and activation is counter-regulated by $CD103^+$cDC *ex vivo*.** (A) Representative histograms showing degrees of proliferation of P25-Tg $CD4^+$ T-cells when co-cultured with either $CD11b^+$cDC alone or $CD11b^+$cDC together with $CD103^+$cDC. Bar graph comparing frequencies of P25-Tg $CD4^+$ T-cells in each generation (B) Bar graph showing levels of IFN-γ in the supernatants of co-cultures measured by ELISA. (C) Representative dotplots and bar graph showing frequencies of P25-Tg $CD4^+$ T-cells producing IFN-γ when co-cultured with either $CD11b^+$cDC alone or $CD11b^+$cDC together with $CD103^+$cDC. (D) Bar graph comparing levels of IL-12 in the supernatants of P25-Tg $CD4^+$ T-cells co-cultured with the indicated cDC subset. Data are expressed as mean±SD of triplicate samples, representative of 2 independent experiments.

**Figure 5. $CD11b^+$cDC-mediated *M.tb* Ag-specific Th1 priming and activation is counter-regulated by $CD103^+$cDC *in vivo*.** (A) Experimental schema (B) Bar graph showing total numbers of adoptively transferred P25-Tg CD4 T-cells in the dLN enumerated as an index for activation of Th1 responses in CD45.1 congenic mice engrafted with either *M.tb*-bearing $CD11b^+$cDC alone, or $CD103^+$cDC alone or both $CD103^+$cDC and $CD11b^+$cDC. Total numbers of transferred P25-Tg CD4 T-cells in the dLN were determined at 3 and 7 days after cDC engraftment. Data are expressed as mean±SD of three mice/group, representative of 2 independent experiments.

**Figure 6. $CD103^+$cDC, but not $CD11b^+$cDC, is a significant source of IL-10 following pulmonary *M.tb* infection.** $CD103^+$ and $CD11b^+$ cDC were purified from the dLN of animals infected with *M.tb* for 14 days. (A) Bar graph showing levels of IL-12 and IL-10 in the supernatants

of $CD103^+$ and $CD11b^+$cDC cultured individually in the presence of *M.tb* antigens. (B) Bar graph showing levels of IL-10 in the supernatants of $CD103^+$cDC and $CD11b^+$cDC cultured individually in the presence of naive *M.tb* Ag-specific P25-Tg $CD4^+$ T-cells purified from naïve P25-Tg mice. (C) Representative zebraplots and bar graph showing frequencies of $CD103^+$cDC or $CD11b^+$cDC producing IL-10 when co-cultured with naive *M.tb* Ag-specific P25-Tg $CD4^+$ T-cells purified from naïve P25-Tg mice. Data are expressed as mean±SD of triplicate samples, representative of 2 independent experiments.

**Figure 7. $CD11b^+$cDC-mediated anti-*M.tb* Th1 activation is counter-regulated by $CD103^+$cDC via IL-10.** (A) $CD11b^+$cDC were purified from the dLN of animals infected with *M.tb* for 14 days and co-cultured with CFSE-labeled *M.tb* antigen-specific P25-Tg $CD4^+$ T-cells for 72-96 hours. Representative histogram showing degrees of proliferation of P25-Tg $CD4^+$ T-cells, measured by extent of CFSE dilution in the absence (Media) or presence of rIL-10. Bar graph comparing frequencies of P25-Tg $CD4^+$ T-cells in each generation. (B) Bar graph showing levels of IFN-γ and (C) Bar graph showing levels of IL-12 in the $CD11b^+$cDC and P25-Tg $CD4^+$ T-cells co-culture supernatants measured by ELISA. (D) $CD11b^+$cDC and $CD103^+$cDC were purified from the dLN of animals infected with *M.tb* for 14 days and co-cultured together with CFSE-labeled *M.tb* antigen-specific P25-Tg $CD4^+$ T-cells for 72-96 hours. Representative histogram showing degrees of proliferation of P25-Tg $CD4^+$ T-cells, measured by extent of CFSE dilution in the absence (Media) or presence of anti-IL-10 receptor mAb (anti-IL10R). Bar graph comparing frequencies of P25-Tg $CD4^+$ T-cells in resting phase (G0) and generations G1-G4 and G5-G7. (E) Bar graph showing levels of IFN-γ and (F) Bar graph showing levels of IL-12 in the co-culture supernatant measured by ELISA. Data are expressed as mean±SD of triplicate samples, representative of 2 independent experiments.

**Figure 1**.

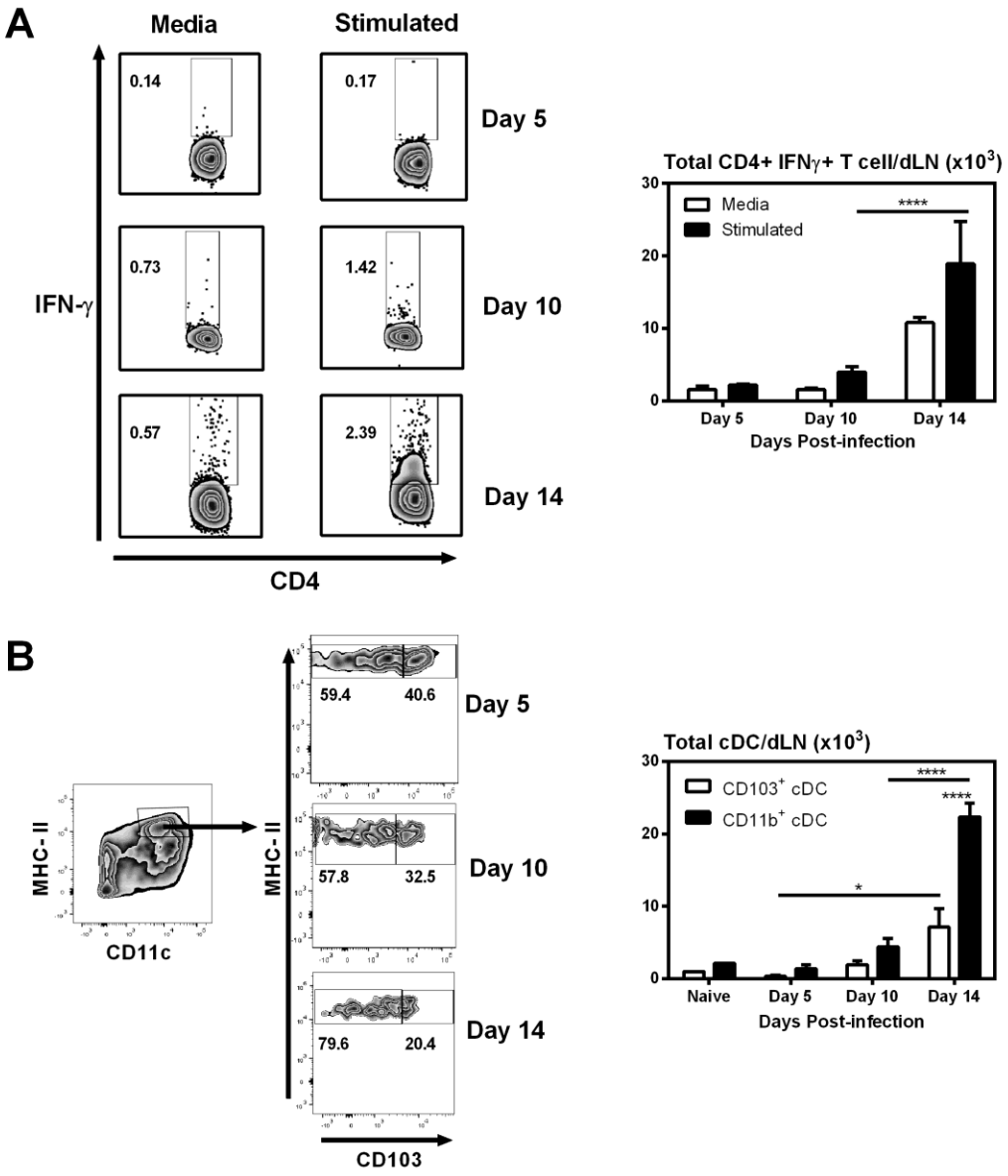

A
Media
Stimulated
0.14
0.17
Day 5
0.73
1.42
Day 10
0.57
2.39
Day 14
IFN-γ
CD4
Total CD4+ IFNγ+ T cell/dLN (x10^3)
Media
Stimulated
****
Day 5
Day 10
Day 14
Days Post-infection
B
59.4
40.6
Day 5
57.8
32.5
Day 10
79.6
20.4
Day 14
MHC- II
CD11c
CD103
Total cDC/dLN (x10^3)
CD103+ cDC
CD11b+ cDC
****
****
*
Naive
Day 5
Day 10
Day 14
Days Post-infection
C
Log10 CFU/million dLN cDC
*
CD103+ DC
CD11b+ DC

**Figure 2**.

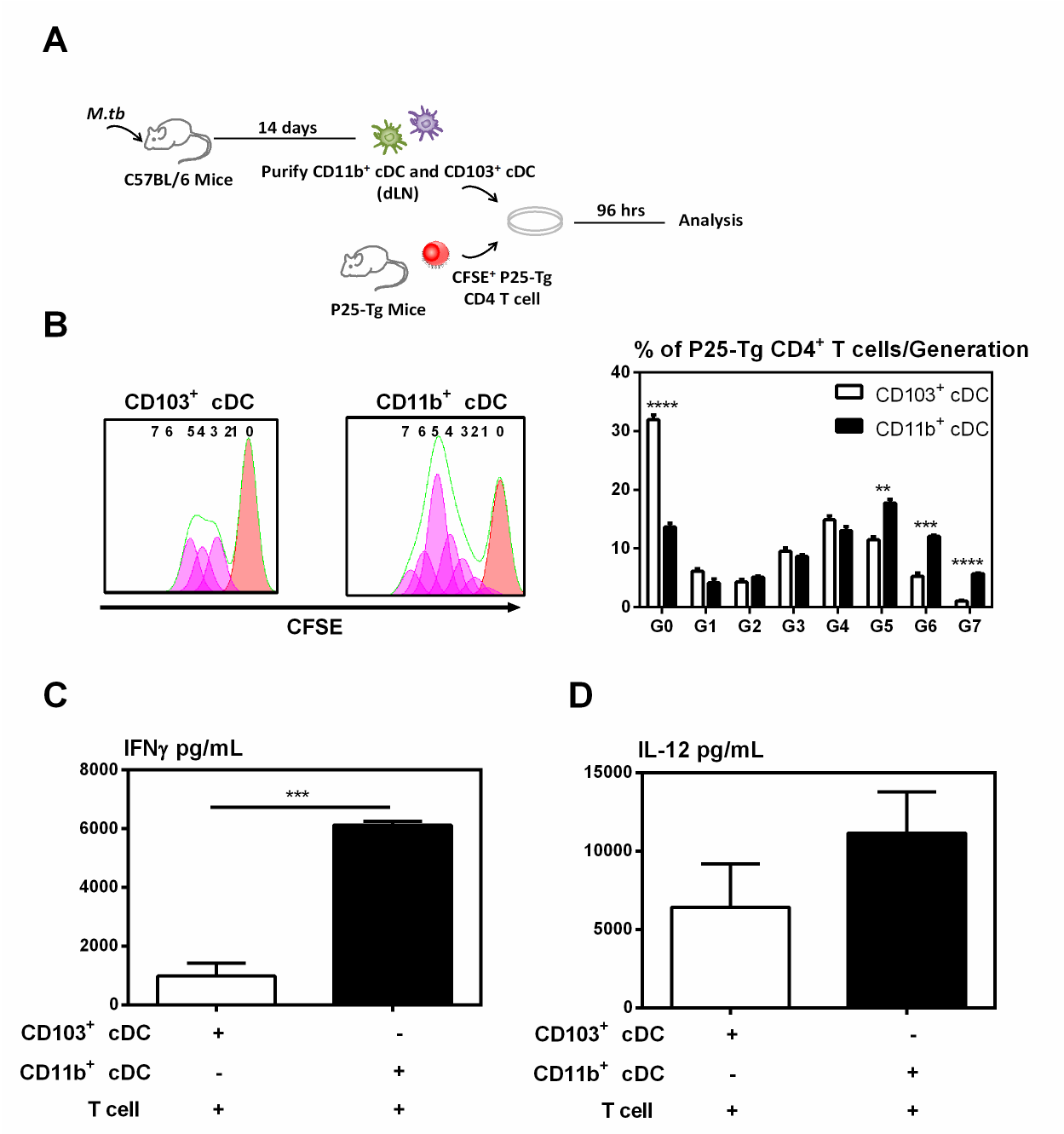

A
M.tb
14 days
C57BL/6 Mice
Purify CD11b+ cDC and CD103+ cDC
(dLN)
96 hrs
Analysis
CFSE+ P25-Tg
CD4 T cell
P25-Tg Mice
B
CD103+ cDC
CD11b+ cDC
7 6 5 4 3 2 1 0
CFSE
% of P25-Tg CD4+ T cells/Generation
CD103+ cDC
CD11b+ cDC
****
**
***
****
G0 G1 G2 G3 G4 G5 G6 G7
C
IFNγ pg/mL
***
CD103+ cDC + -
CD11b+ cDC - +
T cell + +
D
IL-12 pg/mL
CD103+ cDC + -
CD11b+ cDC - +
T cell + +

**Figure 3**.

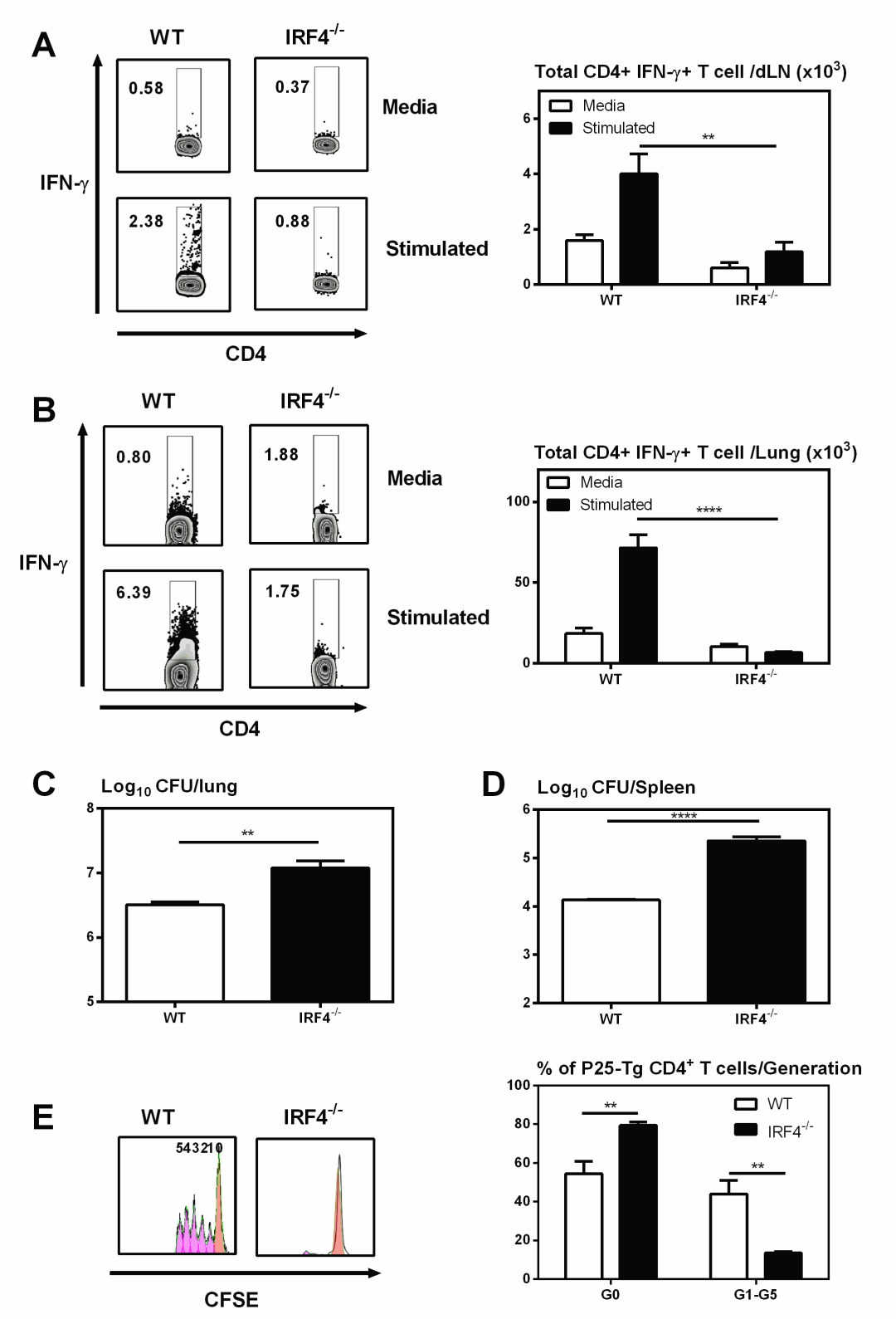

A
WT
IRF4-/-
0.58
0.37
Media
IFN-γ
2.38
0.88
Stimulated
CD4
Total CD4+ IFN-γ+ T cell /dLN (x10^3)
Media
Stimulated
**
WT
IRF4-/-
B
WT
IRF4-/-
0.80
1.88
Media
IFN-γ
6.39
1.75
Stimulated
CD4
Total CD4+ IFN-γ+ T cell /Lung (x10^3)
Media
Stimulated
****
WT
IRF4-/-
C
Log10 CFU/lung
**
WT
IRF4-/-
D
Log10 CFU/Spleen
****
WT
IRF4-/-
E
WT
IRF4-/-
543210
CFSE
% of P25-Tg CD4+ T cells/Generation
WT
IRF4-/-
**
**
G0
G1-G5

**Figure 4**.

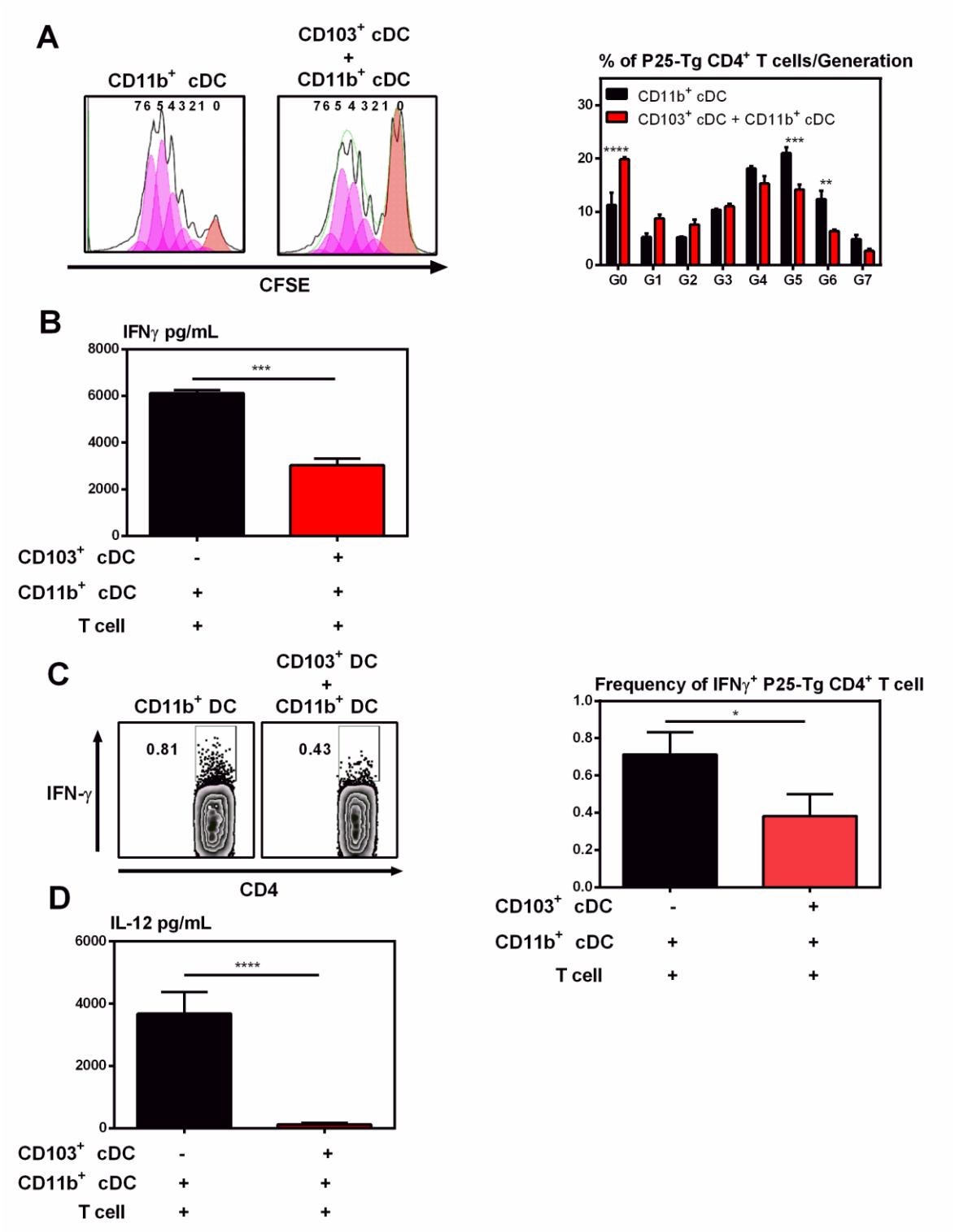

A
CD11b+ cDC
CD103+ cDC + CD11b+ cDC
7 6 5 4 3 2 1 0
CFSE
% of P25-Tg CD4+ T cells/Generation
CD11b+ cDC
CD103+ cDC + CD11b+ cDC
****
***
**
G0 G1 G2 G3 G4 G5 G6 G7
B
IFNγ pg/mL
***
CD103+ cDC - +
CD11b+ cDC + +
T cell + +
C
CD103+ DC + CD11b+ DC
CD11b+ DC
0.81
0.43
IFN-γ
CD4
Frequency of IFNγ+ P25-Tg CD4+ T cell
*
CD103+ cDC - +
CD11b+ cDC + +
T cell + +
D
IL-12 pg/mL
****
CD103+ cDC - +
CD11b+ cDC + +
T cell + +

**Figure 5**.

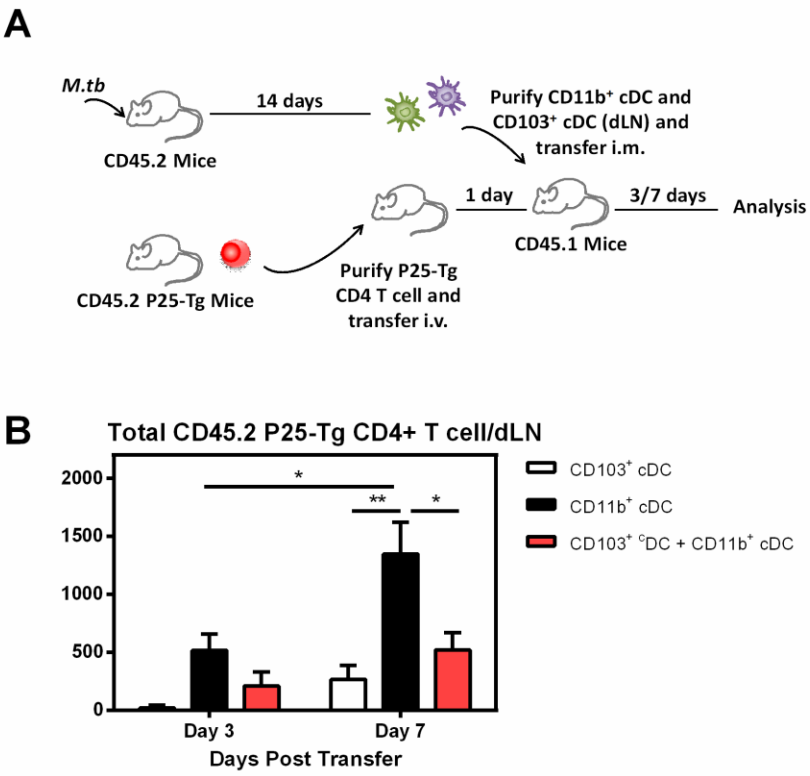

A
M.tb
14 days
CD45.2 Mice
Purify CD11b+ cDC and CD103+ cDC (dLN) and transfer i.m.
1 day
3/7 days
Analysis
CD45.1 Mice
CD45.2 P25-Tg Mice
Purify P25-Tg CD4 T cell and transfer i.v.
B
Total CD45.2 P25-Tg CD4+ T cell/dLN
2000
1500
1000
500
0
*
**
*
Day 3
Day 7
Days Post Transfer
CD103+ cDC
CD11b+ cDC
CD103+ cDC + CD11b+ cDC

**Figure 6**.

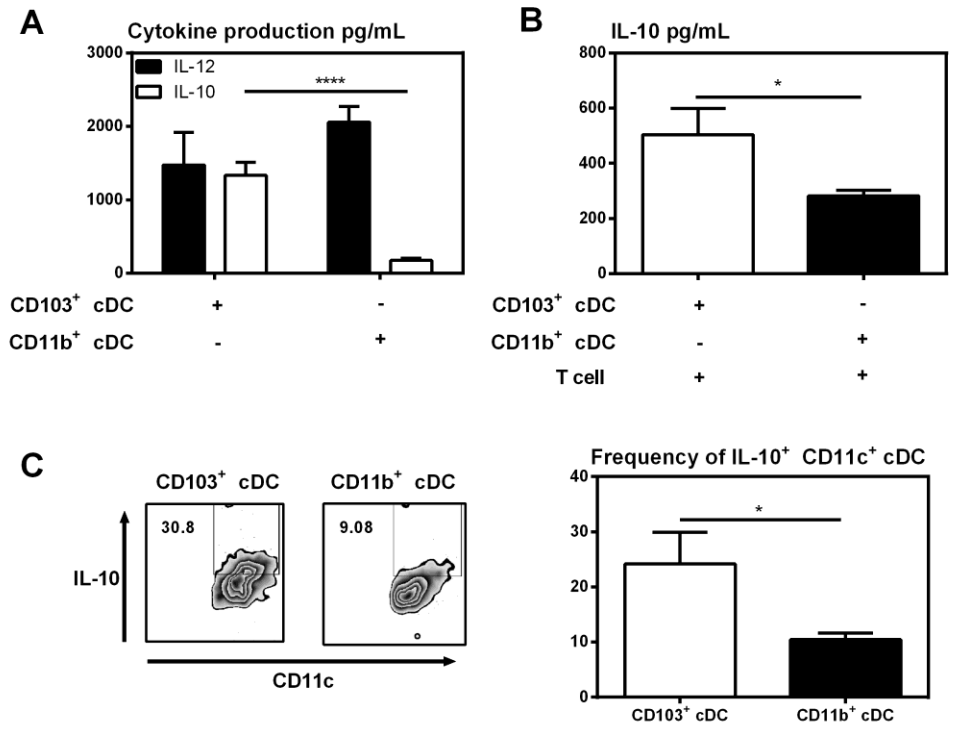
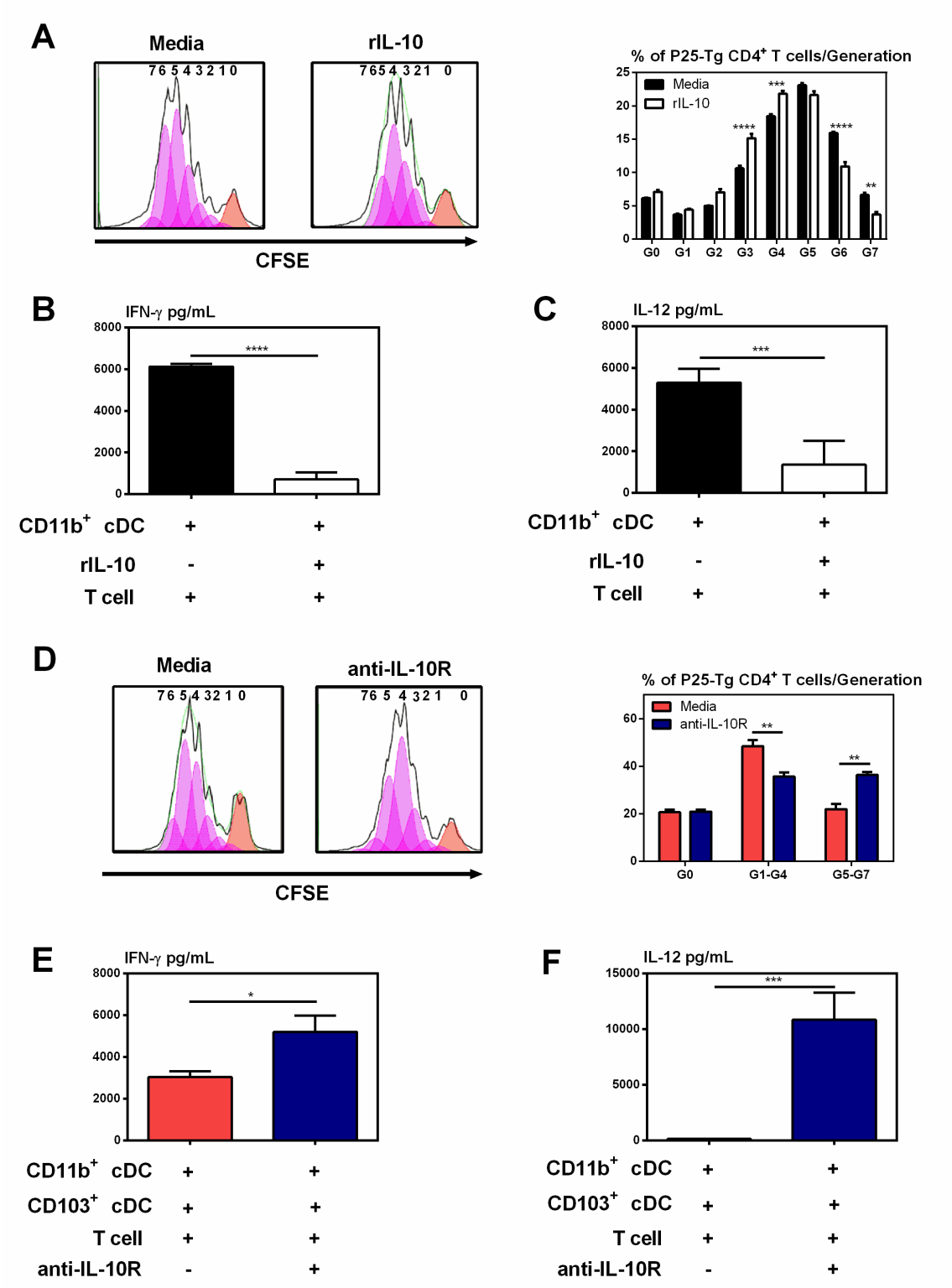

A
Cytokine production pg/mL
IL-12
IL-10
****
3000
2000
1000
0
CD103+ cDC + -
CD11b+ cDC - +
B
IL-10 pg/mL
*
800
600
400
200
0
CD103+ cDC + -
CD11b+ cDC - +
T cell + +
C
CD103+ cDC
CD11b+ cDC
30.8
9.08
IL-10
CD11c
Frequency of IL-10+ CD11c+ cDC
*
40
30
20
10
0
CD103+ cDC
CD11b+ cDC

**Figure 7**.


A
Media
rIL-10
CFSE
% of P25-Tg CD4+ T cells/Generation
Media
rIL-10
G0 G1 G2 G3 G4 G5 G6 G7
B
IFN-γ pg/mL
CD11b+ cDC + +
rIL-10 - +
T cell + +
C
IL-12 pg/mL
CD11b+ cDC + +
rIL-10 - +
T cell + +
D
Media
anti-IL-10R
CFSE
% of P25-Tg CD4+ T cells/Generation
Media
anti-IL-10R
G0 G1-G4 G5-G7
E
IFN-γ pg/mL
CD11b+ cDC + +
CD103+ cDC + +
T cell + +
anti-IL-10R - +
F
IL-12 pg/mL
CD11b+ cDC + +
CD103+ cDC + +
T cell + +
anti-IL-10R - +

## Supplementary Methods

### Mononuclear cell isolation from the lymph node

The lungs, spleen and lymph nodes were digested as previously described [1-3]. The lungs were cut into small pieces and digested with 150U of collagenase type I from Gibco (Grand Island, NY, USA) in Hanks Buffer for 1 hr at 37˚C with agitation. Lymph nodes were teased apart on a petri-dish with 23-gauge needles and digested with 1 µg/mL of Collagenase Type II from Worthington (Lakewood, NJ, USA) in RPMI for 1 hr at 37˚C with agitation. The digested lungs and lymph nodes, as well as spleens, were then crushed through a 100-µm filter from BD Falcon (Franklin Lakes, NJ, USA). Lungs and spleens were treated with ACK Lysis buffer for 2 minutes to remove all red blood cells. Cells were then resuspended in RPMI supplemented with 10% FBS, 1% Pen-Strep and 1% L-Glutamine (cRPMI). Isolated cells were resuspended in cRPMI and counted using Scepter cell counter from Millipore (Etobicoke, ON, CA).

### DC purification

Mononuclear cells were isolated from the mediastinal lymph nodes of *M.tb*H37Rv infected mice at day 14 post-infection as described. Dendritic cells were then purified using a multi-sort approach according to manufacturer's instructions. Briefly, cells were labelled with CD11c-APC from BD Bioscience (San Jose, CA, USA) and CD103-Biotin from eBiosciences (San Diego, CA, USA), after which $CD11c^{hi}$ cells were purified using 4µL of anti-APC magnetic beads from the anti-APC MultiSort kit from Miltenyi Biotec (Auburn, CA, USA) according to manufacturer's recommendations. Purity was consistently greater than 90% as assessed by flow cytometry. Magnetic beads were then removed according to manufacturer's instructions, after which $CD11c^{hi}$ fraction was further separated into $CD103^{+}$ ($CD103^{+}$cDC) and $CD103^{-}$ ($CD11b^{+}$cDC) populations

using 10µL of the anti-biotin beads from the anti-Biotin MultiSort kit from Miltenyi Biotec as described. DC subsets were enumerated using the Scepter cell counter from Millipore (Etobicoke, ON, CA). Expression of CD103 on the $CD103^+$ fraction as well as the expression of CD11b on the $CD103^-$ fraction is described in Figure S6.

**Transgenic T cell purification, CFSE labelling, and *in vivo* and *ex vivo* antigen presentation assays**

The mononuclear cells isolated from the spleen and lymph nodes of P25-Tg mice were subjected to the purification of $CD4^+$ T cells by using a Mouse CD4 T cell negative selection kit according to manufacturer's instructions (Stemcell Technologies, Vancouver, CA). Purity (>90%) was verified by flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA). Cells were resuspended to a concentration of $10^7$ cells/mL and labelled with Carboxyfluorescein succinimidyl ester (CFSE) from Invitrogen (Burlington, ON, Canada) at a final concentration of 5µM. Cells were washed twice with 5% FBS/PBS to remove excess CFSE, after which they were resuspended in PBS. Cells were then either used in antigen presentation assays or adoptively transferred intravenously (i.v.) into animals. For i.v. adoptive transfer, approximately $2\text{-}3x10^6$ cells were resuspended in 200µL of PBS and transferred into mice via tail-vein injection. After 24 hrs mice were infected with *M.tb* as described.

In other experiments, CFSE labelled $CD4^+$ T cells were also used to assess antigen presentation capabilities of each cDC subset. $CD103^+$ cDC and $CD11b^+$ cDC were isolated as described and paired with P25-Tg $CD4^+$ T cells at a ratio of 1:2 cDC to T cell ($1x10^5$ DC to $2x10^5$ T cells) for 72-96 hours at 37°C. To assess the priming potential of each cDC subset, in some assays P25-Tg $CD4^+$ T cells were labelled with 0.5mM CFSE as described, and proliferation of T

cells was assessed using FlowJo. Th1 polarization was assessed through intracellular cytokine staining for IFN-γ and IL-10. In addition, cell culture supernatants were also collected, and IFN-γ, IL-12 and IL-10 levels were measured using the respective duoset kit from R&D Systems (Burlington, ON, Canada).

**Supplementary Figures**

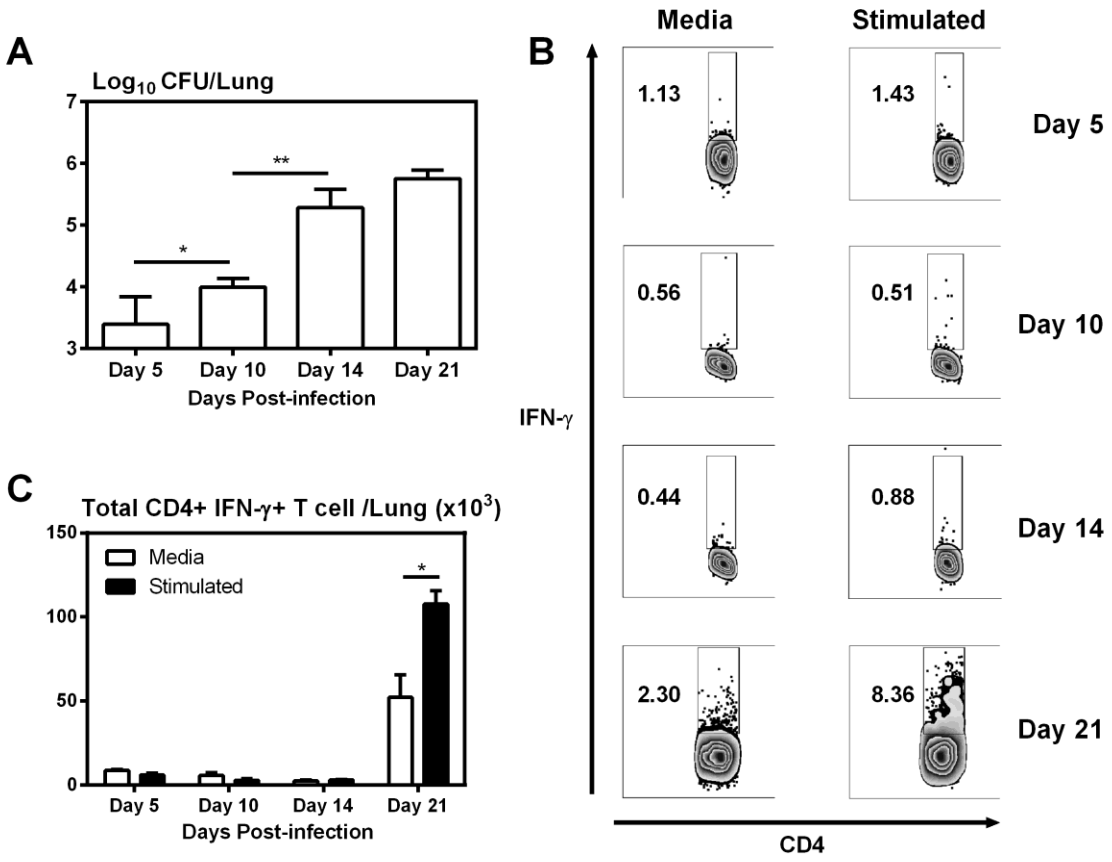
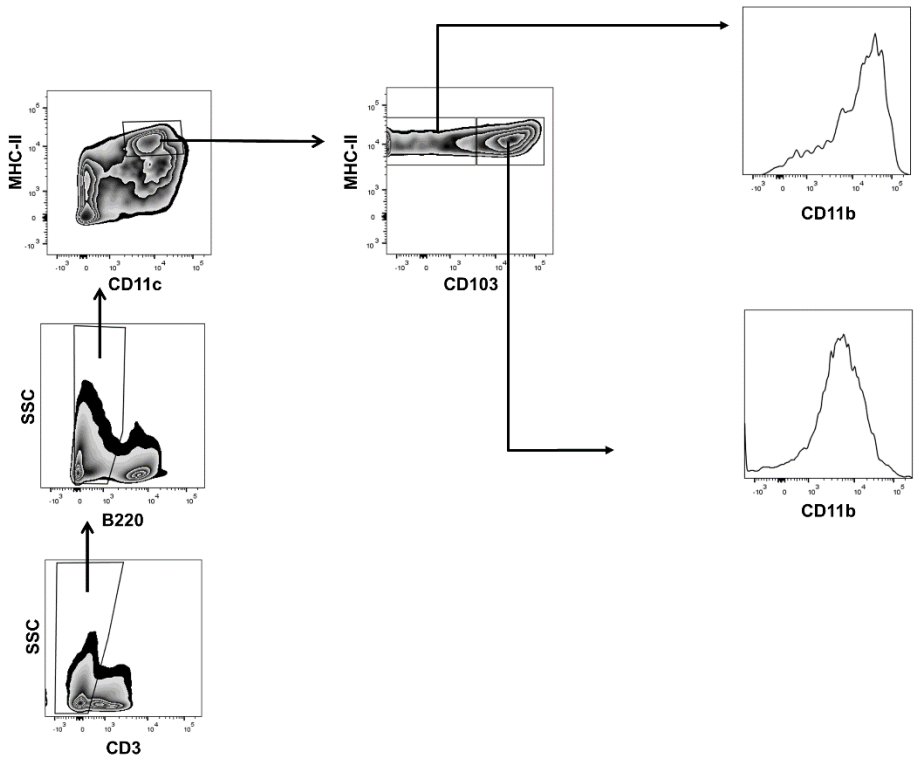



**Figure S1. Ag-specific Th1 cell recruitment to the lung and bacterial control are significantly delayed following pulmonary *M.tb* infection.** (A) Bar graph showing levels of *M.tb* infection (CFU–colony forming units) in the lung at the indicated timepoints following pulmonary *M.tb* infection. (B) Representative dotplots showing frequencies of Ag-specific $CD4^{+}IFN\text{-}\gamma^{+}$ T-cells in the lung at the indicated timepoints following pulmonary *M.tb* infection. (C) Bar graph showing numbers of Ag-specific $CD4^{+}IFN\text{-}\gamma^{+}$ T-cells in the lung at the indicated timepoints following pulmonary *M.tb* infection. Data are presented as mean±SD of five mice (A) or three mice (B/C) per timepoint and group, representative of 3 independent experiments.




**<u>Figure S2.</u> Gating Strategy for the identification of CD11b$^{+}$cDC and CD103$^{+}$cDC in the dLN.** Gating strategy used to identify CD103$^{+}$cDC and CD11b$^{+}$cDC in the dLN, as described [4]. In brief, cells were first gated to exclude CD3$^{+}$ T-cells and B220$^{+}$ B cells, after which CD11c$^{hi}$ and MHCII$^{hi}$ populations were identified. CD103$^{+}$cDC and CD11b$^{+}$cDC were separated from within that population based on the expression of CD103, after which CD11b expression was examined within each population for confirmation.

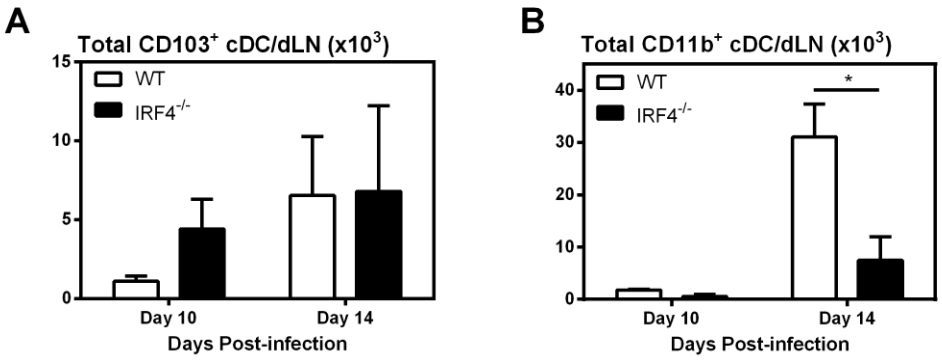



**Figure S3. Differential levels of conventional dendritic cell subsets in the dLN of IRF4$^{-/-}$ animals following pulmonary *M.tb* infection**. Bar graphs representing the total numbers of CD11b$^{+}$cDC (A) and CD103$^{+}$cDC (B) subsets in the dLN at the indicated timepoints following pulmonary *M.tb* infection (see Fig.S3 for gating strategy). Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

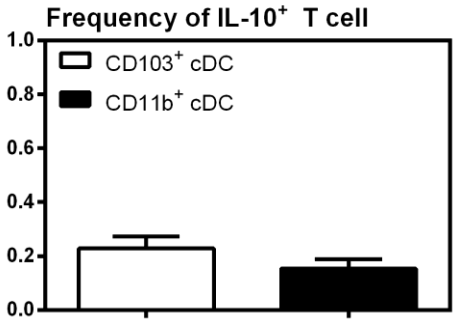
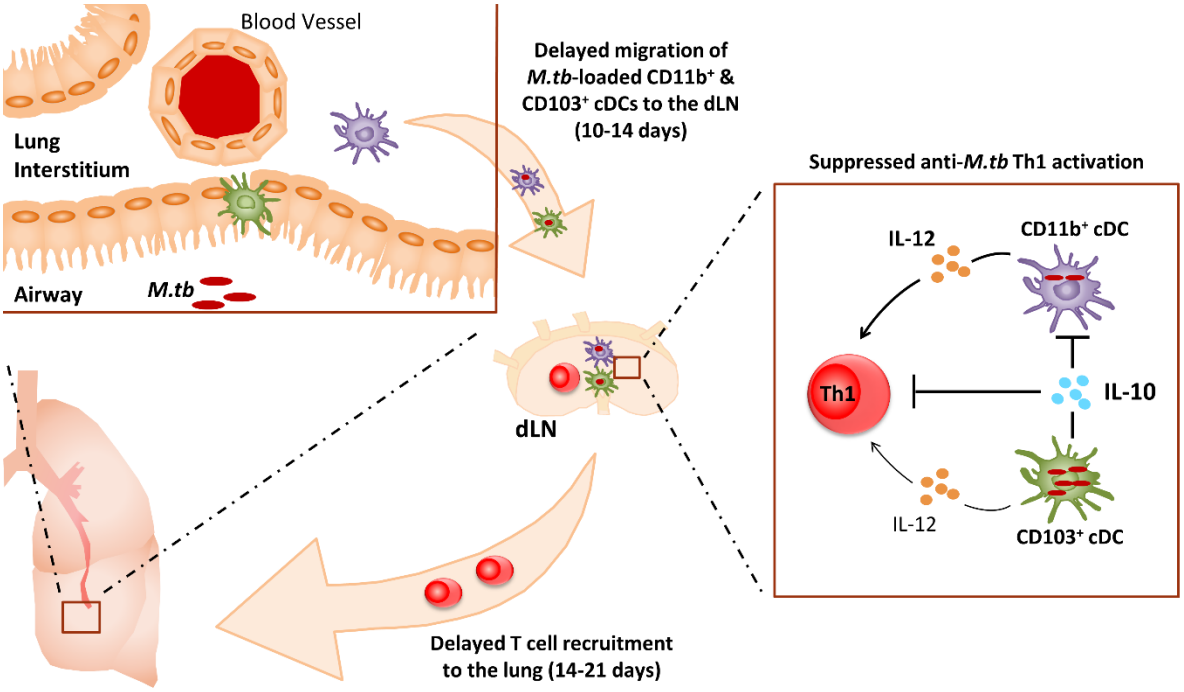
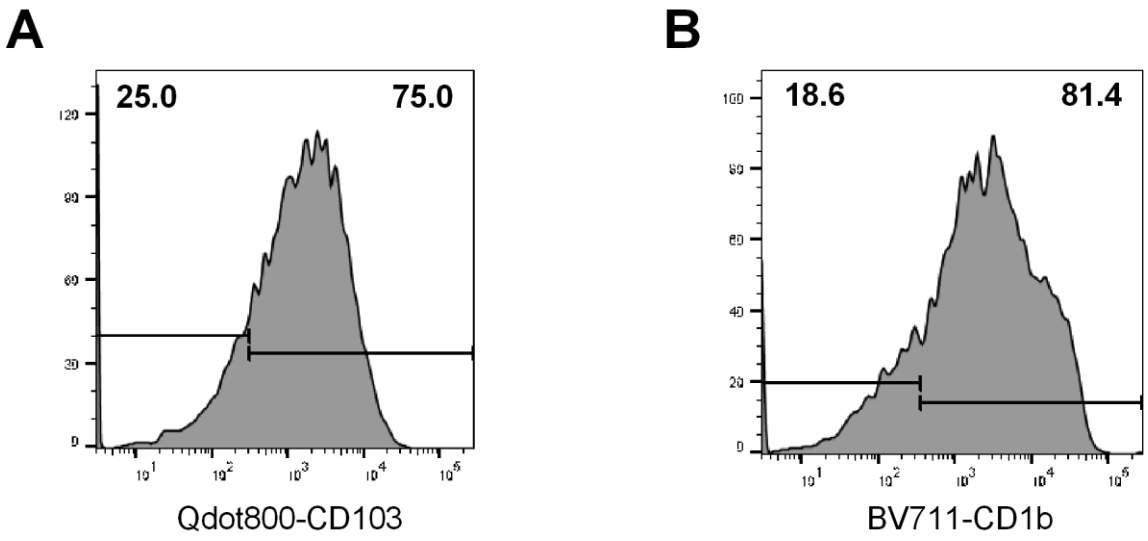



**Figure S4. Similar small frequencies of P25-Tg CD4$^{+}$ T-cells expressing IL-10**. Bar graph showing frequencies of IL-10$^{+}$ P25-Tg CD4$^{+}$ T-cells cultured either with CD103$^{+}$cDC or CD11b$^{+}$cDC in the presence of *M.tb* Ag85 complex for 72-96 hours. Data are expressed as mean±SD of triplicate samples/group, representative of 2 independent experiments.




**<u>Figure S5.</u> Conceptual illustration of identified novel cellular and molecular mechanisms underlying delayed Th1 immunity in the lung following pulmonary *M.tb* infection**. The initiation of anti-*M.tb* Th1 immunity is dependent on initial innate immune responses. While the conventional or classic dendritic cells (cDC) are believed to be critical to the initiation of anti-*M.tb* Th1 responses, the precise roles and mechanisms of cDC subsets in anti-*M.tb* Th1 immunity have remained poorly defined. Our study finds that following pulmonary *M.tb* infection, both infected $CD11b^+$cDC and $CD103^+$cDC co-translocate into the lung dLN but their migration is delayed for 10-14 days. The bacterial load in $CD103^+$cDC is relatively higher than in $CD11b^+$cDC perhaps due to their preferential intraepithelial positioning in the lung. In the dLN, compared to $CD103^+$cDC, $CD11b^+$cDC are the major cellular source of Th1-polarizing cytokine IL-12 and the principal cDC driving *M.tb* Ag-specific Th1 priming and activation. However, such $CD11b^+$cDC-mediated anti-*M.tb* Th1 activation is subject to inhibition by $CD103^+$cDC via their production of

a potent immune suppressive cytokine IL-10 in the dLN. In comparison, $CD11b^{+}$cDC produce little IL-10. $CD103^{+}$cDC-derived IL-10 counter-regulates $CD11b^{+}$cDC-driven Th1 activation by inhibiting IL-12 production from $CD11b^{+}$cDC on one hand, and by directly acting on Th1 cells on the other. Taken together, the delayed anti-*M.tb* Th1 immunity in the lung results from delayed transmigration of antigen-bearing cDC subsets from the lung to the dLN and activate suppression of $CD11b^{+}$cDC-mediated Th1 activation by $CD103^{+}$cDC in the dLN. Our findings shed new mechanistic insights into the distinct roles of cDC subsets in immune regulation of Th1 activation against a clinically important intracellular bacterial pathogen and shall help develop effective prophylactic and therapeutic strategies.




**<u>Figure S6</u> – Purity of $CD103^{+}$cDC and $CD11b^{+}$cDC populations isolated from the lung dLN 14 days post *M.tb* infection**. Mononuclear cells were isolated from the lung dLN day 14 post-pulmonary *M.tb* infection and labeled with APC-CD11c and Biotin-CD103 antibodies, after which the $CD11c^{hi}$ populations were purified using a MACS Multisort system directed against APC. After removal of the magnetic beads, the $CD11c^{hi}$ population was further divided into two $CD103^{+}$

($CD103^+$cDC) and $CD103^-$ ($CD11b^+$cDC) fractions using a second Multisort directed against biotin. Over 75% of the population in the $CD103^+$ fraction expressed bright CD103 (A) and over 80% of the $CD103^-$ fraction expressed high levels of CD11b (B).

## References


1. Jeyanathan M, Thanthrige-Don N, Afkhami S, Lai R, Damjanovic D, Zganiacz A *et al.* Novel chimpanzee adenovirus-vectored respiratory mucosal tuberculosis vaccine: overcoming local anti-human adenovirus immunity for potent TB protection. *Mucosal Immunol* 2015; **8**(6)**:** 1373-1387.

2. Lai R, Jeyanathan M, Shaler CR, Damjanovic D, Khera A, Horvath C *et al.* Restoration of innate immune activation accelerates Th1 cell priming and protection following pulmonary mycobacterial infection. *Eur J Immunol* 2014.

3. Horvath CN, Shaler CR, Jeyanathan M, Zganiacz A, Xing Z. Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: A critical role of airway luminal T cells. *Mucosal Immunol* 2012; **5**(4)**:** 420-431.

4. Ballesteros-Tato A, Leon B, Lund FE, Randall TD. Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. *Nat Immunol* 2010; **11**(3)**:** 216-224.

# Chapter 5. What is the relative contribution of innate and adaptive immune responses in the early phase of *M.tb* infection in respiratory AdHuAg85A immunized hosts?

**Title of Manuscript**: Mucosal imprinting of host innate immunity following AdHuAg85A vaccination protects against pulmonary *M.tb* infection independently of T cell responses.

**Rocky Lai**, Sam Afkhami, Mangalakumari Jeyanathan, Anna Zganiacz, Zhou Xing

Prepared for submission to *Mucosal Immunology*

Expected submission September 2017

In this chapter, we examine the relative contribution of both the innate and adaptive immune compartment in protection against pulmonary *M.tb* infection following respiratory mucosal vaccination with AdHuAg85A. Although we have previously demonstrated that the superiority of respiratory mucosal vaccination is associated with the induction of Ag-specific Th1 responses within the lungs, the relative contribution of the innate immune compartment to host anti-TB immunity is not clear. Given that it has been previously demonstrated that mucosal vaccination with AdHuAg85A can directly impact innate immune cells independently of T cell responses, we seek to elucidate their relative contribution in vaccine induced immune-protective outcomes.

Please refer to the *Declaration of Academic Achievement* for author contribution details.

**Mucosal imprinting of host innate immunity following AdHuAg85A vaccination protects against pulmonary *M.tb* infection independently of T cell responses.**

Rocky Lai, Sam Afkhami, Manglakumari Jeyanathan, Anna Zganiacz, Zhou Xing[1*]

**Affiliations:**

[1] McMaster Immunology Research Centre, M. G. DeGroote Institute for Infectious Disease Research, and Department of Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario, Canada.

*__Address correspondence to__: Dr. Zhou Xing, Department of Pathology and Molecular Medicine, and McMaster Immunology Research Centre, McMaster University, 1280 Main Street West, Room MDCL-4012, Hamilton, Ontario L8S 4K1, Canada.

E-mail address: xingz@mcmaster.ca

**Abstract**

The induction of Th1 immunity has long been understood to be critical to host immunity against pulmonary *M.tb* infection. As such, current TB vaccine strategies have been designed around the basis of eliciting a robust Th1 immune response. In particular, the mucosal administration of AdHuAg85A has been demonstrated to be effective in the induction of anti-TB Th1 immunity and providing superior protection over parenterally administered vaccines. However, the relative contribution of T cell responses and innate immune responses remain unclear. Using a T cell depletion approach, we demonstrate that mice remain protected following respiratory mucosal vaccination with AdHuAg85A in the absence of T cells for at least 7 days of pulmonary *M.tb* infection. We find that this is due to an enhanced killing capacity of the innate immune compartment. Specifically, we find that respiratory mucosal vaccination with AdHuAg85A enhances the activation of alveolar macrophages (AM), and is correlated to their enhanced killing capacity towards *M.tb*. Furthermore, we find that SCID mice, which are deficient in T cells from birth, are also protected from *M.tb* during the first 7 days post infection following respiratory mucosal vaccination. Our study demonstrates that respiratory mucosal vaccination directly modulates the host innate immune compartment and enhances their functionality, contributing towards the early phase of protection against *M.tb* independent of T cell responses.

## Introduction

To this day, pulmonary tuberculosis (TB) continues to be a leading cause of global morbidity and mortality due to infectious disease, with an estimated 10.4 million new cases and 1.8 million deaths worldwide each year (*1*). Although the currently approved TB vaccine, BCG, has proven to be successful in protecting against disseminated childhood forms of TB, it fails to protect against adult pulmonary TB. As a result, over one third of the world remains lately infected with TB (*2*). Thus, there is a pressing need to develop a novel vaccine that will be able to boost existing BCG-mediated responses.

To date, there are over a dozen candidate TB vaccines at various stages of testing in the clinical trial pipeline, either designed to replace BCG or boost existing BCG responses. Viral vectored vaccines such as AdHuAg85A have proven particularly successful in both animal models and clinical trial testing (*3*, *4*). However, irrespective of the vaccine formulation, there is mounting evidence demonstrating that mucosal intranasal (i.n.) vaccination provides superior protection against pulmonary tuberculosis in comparison to parenteral vaccination. In particular, we have demonstrated that the ability of AdHuAg85A to protect against pulmonary *M.tb* is due to the induction Ag-specific T cells in the lung airways and tissue following i.n. administration (*5*, *6*).

However, it has come to recent attention that T cells may not be the sole contributing factor in the superiority of mucosal vaccination. Although much of vaccine research into the generation of anti-TB immunity is focused on the induction of Ag-specific T cell responses, increasing evidence has suggested that the functionality of innate APC populations may also be critical factors in determining the protective efficacy of a given vaccine formulation. The earliest evidence of this stems from a study of a TB outbreak on an US naval ship where they found 13

individuals who remained protected despite the absence of T cell immunity for at least 6 months post exposure (*7*), suggesting that the protection was mediated by the host innate immunity.

Innate immune cells reside within both the lung airway and interstitium and are among the first to encounter and respond to foreign stimuli. Alveolar macrophages (AM) are particularly important as they reside within the lung airway (*8*), and represents one of the first targets of infection by *M.tb (9)*. There is substantial evidence demonstrating the modulation of the local innate immune responses by respiratory mucosal vaccination. We have demonstrated that VSV is inferior as an TB vaccine platform due to the induction of type I IFN, which leads to an impairment in the *M.tb* killing capacity of lung APC despite inducing a more potent adaptive T cell response (*10*). In contrast, adenoviral vectors have been well established to induce potent inflammatory responses within the local innate compartment when administered mucosally while inducing relatively little type I IFN. Together, this indicates that while the induction of adaptive immunity at the site of infection by mucosal vaccination is a critical component to its efficacy, the influence it has on the local innate immune response must also be considered.

In this study we determine the relative contribution of both the adaptive and innate immunity during the early phase of *M.tb* infection. We show that respiratory mucosal vaccination with AdHuAg85A confers protection against pulmonary *M.tb* for at least 7 days following infection, even in the absence of T cells. Our evidence suggests that this is due to enhanced activation of lung alveolar macrophages, leading to their enhanced killing capacity. Our study thus demonstrates that T cells may not be required for early protection against *M.tb*. Furthermore, we have evidence to suggest that T cells are not required to imprint local lung innate cells.

## Results

### T cells are not required for early protection against pulmonary *M.tb* infection following intranasal vaccination with AdHuAg85A

To begin addressing the contribution of the innate immune response in protection against pulmonary *M.tb* following intranasal (i.n.) vaccination with AdHuAg85A, we depleted both $CD4^+$ and $CD8^+$ T cells prior to infection with *M.tb*. At 4 and 8 weeks post vaccination, a select group of naïve and immunized mice were treated with CD4 and CD8 depleting antibodies to deplete T cells. One day after depletion mice were infected with *M.tb*H37Rv i.n., after which bacterial burden was assessed 7 days post infection (Figure 1A). As expected, mice that were i.n. immunized with AdHuAg85A for both 4 weeks (Figure 1B) and 8 weeks (Figure 1C) had greater protection than unimmunized controls. Interestingly, we also saw a similar level of protection in AdHuAg85A immunized mice that were depleted of $CD4^+$ and $CD8^+$ T cells at both timepoints (Figure 1). Together, this suggests that T cells are not required for protection during the early phase of *M.tb* infection in intranasally immunized mice, and that the protective phenotype persists for at least 8 weeks post vaccination.

### Innate immune cells have superior killing capacity following intranasal vaccination with AdHuAg85A

Although we found that mice were protected in spite of the absence of T cells for at least 7 days post infection, the mechanism by which these mice remained protected against *M.tb* remained unclear. To begin addressing whether the enhanced protection observed even in the absence of T cells was due to an increase in anti-TB killing capability by the local lung innate immune cells, lung cells were purified from naïve or i.n. AdHuAg85A immunized mice 4 weeks post vaccination, after which $CD11c^+$ and $CD11b^+$ cells innate immune cells were purified and

pooled to get a representation of the lung innate immune cells. Approximately 1 million pooled $CD11c^+/CD11b^+$ cell s were plated onto a 24 well plate, after which *M.tb*H37Rv was added to the cells in a 1:1 cell to bacteria ratio. After 4 hours, cells were washed with PBS to remove extracellular bacteria, and bacterial burden was assessed at 0hr, 24hr and 48 hr post wash. As expected, we found that the $CD11c^+/CD11b^+$ cells isolated from AdHuAg85A-vaccinated animals had superior killing capacity in comparison to naïve controls, as demonstrated by the higher bacterial burden detected in the naïve cell cultures (Figure 2). Together, this suggests that AdHuAg85A imprints local innate immune cells and enhances their *M.tb* bactericidal capacity in comparison to their naïve counterparts.

**Enhanced activation of alveolar macrophages following intranasal vaccination with AdHuAg85A**

Although we have determined that innate immune cells from AdHuAg85A immunized animals had superior anti-TB killing capacity, given the heterogeneity within the $CD11c^+/CD11b^+$ innate immune cells we sought to identify the specific population responsible for the enhanced protection. Balb/c mice were immunized with AdHuAg85A for 4 weeks, at which a group of AdHuAg85A immunized mice were depleted of $CD4^+$ and $CD8^+$ T cells as described. All mice were subsequently infected with *M.tb* as described (Figure 1A). Lung mononuclear cells were isolated 7 days post *M.tb* infection, and innate immune cells were identified according to the expression of cell surface markers described in **Table 1**. No differences in the absolute number of neutrophils, natural killer (NK) cells (Figure S1) or alveolar macrophages (AM) was observed in the lung (Figure 3A). However, alveolar macrophages of AdHuAg85A immunized mice had a higher level of activation in comparison to naive control, as evidenced by the increased expression of MHC-II in comparison to naïve

controls. Furthermore, this phenotype remained unchanged even in the absence of T cells (Figure 3B and C). Although we saw an increase in the number of dendritic cells (DC) in AdHuAg85A immunized mice, this increase was abolished in T cell depleted animals (Figure S2). Together, this suggests that the enhanced protection observed during the early phase of *M.tb* infection following AdHuAg85A vaccination is due to an increased activation of the AM population.

**AdHuAg85A is able to mediate protection of SCID mice from pulmonary *M.tb* infection**

Our data suggests that AM are the key population responsible for the enhancement of early protection against *M.tb* in an AdHuAg85A immunized host. However, the mechanism responsible for imprinting this population remains unclear. Although we had previously demonstrated that T cells are not required for early protection in AdHuAg85A immunized mice (Figure 1), T cells were not depleted until 4 or 8 weeks post vaccination. As such, the presence of Ag-specific T cells may have modulated the functionality of the innate immune cells at the lung. In order to investigate the requirement of T cells prior to infection with *M.tb*, we immunized SCID mice, which are deficient in T and B cells, either with AdHuAg85A or PBS control intranasally. At 4 weeks post vaccination, we infected these mice with *M.tb*H37Rv and assessed protection 7 days post infection. Although these mice were not protected to the same level as wildtype Balb/c mice (Figure 1), we nevertheless saw enhanced protection in SCID mice immunized with AdHuAg85A (Figure 4A). Given the critical role of IFN-γ and TNF in anti-TB immunity, we also assessed the levels of these cytokines in the lung homogenates. Although no differences were observed in IFN-γ between naïve and AdHuAg85A immunized mice (Figure 4B), there was a significant increase in TNF-α levels in AdHuAg85A immunized mice (Figure 4C). Together, these data demonstrate that i.n. AdHuAg85A vaccination can modulate the innate compartment independent of T cell responses.

## Discussion

TB continues to be the leading infectious cause of global morbidity and mortality despite the availability of BCG as a clinically approved vaccine. The inability of BCG to provide complete protection against TB speaks to the urgent need for the development of novel vaccine formulations to either enhance or replace BCG. Traditionally, vaccine development has generally been focused on the induction of a robust anti-TB T cell mediated responses (*3*, *11*), and viral vectored vaccines such as AdHuAg85A have proven successful in that regard for both animal and human models (*4*, *12*), particularly when delivered via the respiratory mucosa.

Although the superiority of respiratory mucosal vaccination has been associated with the presence of antigen-specific T cells in the lung airway and interstitium (*5*, *6*), one aspect that has remained unexplored is the involvement of the innate immune compartment. Given that the mucosal route of delivery would also result in direct modulation of the local lung innate immune compartment, we sought to understand how this may have contributed to host defence against *M.tb* independently of T cell responses. We find that following respiratory mucosal vaccination with AdHuAg85A, T cells are not required during the initial phase of pulmonary *M.tb* infection (Figure 1). Furthermore, we find that the innate immune cells in these mice have superior killing capacity compared to unimmunized animals (Figure 2). This superior protection observed in vaccine-exposed innate cells may be due to the enhanced activation in AM, as these cells demonstrate higher levels of activation compared to naïve controls (Figure 3). Although mucosal vaccination with AdHuAg85A also led to an increase in DC recruitment, CD4/CD8 depleting antibodies also resulted in a decreased number of these cells in the lung in addition to $CD4^+/CD8^+$ T cells (Figure S2). However, given that these mice remained protected against *M.tb*, it is likely that these cells are not critical to early host defence against *M.tb*. Although we

did not see any differences in the number of NK cells or neutrophils (Figure S1), we cannot dismiss their involvement in anti-TB immunity as their functionality and activation status has not been assessed.

The activation of the innate compartment by respiratory mucosal vaccination likely contributes to the superior protection observed in comparison to parenteral vaccination. Adenoviruses are highly immunogenic, and the delivery of such a powerful adjuvant through the respiratory mucosa would likely serve to activate the local environment and enhance the inflammatory signals needed to draw Ag-specific T cell responses to the site of infection. This is consistent with the observation that TNF-α levels were increased in SCID mice following mucosal vaccination with AdHuAg85A (Figure 4C). We have previously demonstrated that the administration of toll-like receptor (TLR) agonist to the lung of intramuscularly immunized mice is able to draw antigen-specific T cells from peripheral sites to the airway lumen and enhance protection against pulmonary *M.tb (15, 16)*. We have also demonstrated that this approach is an effective method to accelerate T cell responses in the lung in an *M.tb*-infected host (*17*). As such, activation of the local innate environment through mucosal vaccination strategies may also serve as an effective means to enhance T cell recruitment to the site of infection where they are needed.

However, we have also demonstrated that respiratory mucosal vaccination can also enhance the bactericidal capabilities of the innate immune compartment, demonstrating a more direct modulation of the local innate cells by the viral vectored vaccines. We have previously demonstrated that although a vesicular stomatitis virus (VSV)-based vector was able to induce a more robust T cell response in comparison to Ad5, animals immunized with VSV were nevertheless more susceptible than Ad5 immunized animals. This was due to the induction of

type I IFN by VSV vector but not the Ad5 vector, which led to an induction of IL-10 on the infected innate cells and reduced their bactericidal capabilities (*10*). Together, this suggests that the lung macrophages are extremely sensitive to modulation by viral vectored vaccines. More importantly, lung macrophages may play a more critical role in controlling *M.tb* during the early stages of the infection than previously thought. Although BCG has not been shown to directly imprint lung macrophages, the use of BCG has nevertheless been shown to have a protective effect against a variety of non-TB heterologous infections even in the absence of T cells (*18, 19*).

We also have evidence suggesting that T cells are not required in the imprinting of the innate compartment. Following respiratory mucosal vaccination with AdHuAg85A, SCID mice were better protected against *M.tb* than naïve controls (Figure 4). Traditionally, infected macrophages are heavily dependent on the production of IFN-γ and TNF-α by recruited antigen-specific $CD4^+$ T cells (*8, 11, 20*), which leads to the upregulation of processes such as nitric oxide production and phago-lysosome fusion which are essential to anti-TB immunity (*9, 21*). While we do see elevated TNF-α levels in vaccinated SCID mice (Figure 4C), we observed no differences in the levels of IFN-γ between naïve and vaccinated mice (Figure 4B), suggesting an IFN-γ independent role in the activation of AM. Others have also demonstrated an IFN-γ independent macrophage activation (*22*), suggesting other mechanisms may be at play.

In summary, we have identified a mechanism for enhanced protection following respiratory mucosal vaccination that is separate from its ability to induce a population of antigen-specific T cells at the site of infection. Our study highlights the importance of the innate immune compartment as a distinct and separate contributor to host defence and provides rationale for targeting this compartment as a complementary strategy to enhance the efficacy of existing vaccine platforms.

## Materials and Methods

### *Mice*

Female Balb/c mice (6-8 weeks old) were purchased from Charles River (Wilmington, MA, USA). All experimental mice were housed within the Level 3 Biosafety Facility at McMaster University. SCID mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All experiments were conducted in accordance with the McMaster University Animal Research Ethics Board.

### *Mycobacterium tuberculosis* infection and AdHuAg85A intranasal vaccination

The *M.tb*H37Rv strain was prepared for infection and delivered as previously described (*16, 17, 23*). Briefly, *M.tb* was grown in 7H9 media supplemented with Middlebrook oleic acid–albumin–dextrose–catalase enrichment, 0.002% glycerol, and 0.05% Tween-80 for 10–15 days, aliquoted, and stored in −70°C until use. Prior to use, *M.tb* was washed twice with PBS containing 0.05% Tween-20, after which it was passed through a 27-gauge syringe to ensure single cell suspension, after which the bacteria was delivered. Mice were infected with either $1x10^4$ *M.tb* intranasally. Bacterial burden was assessed at each experimental timepoint by plating serial dilutions of lung or spleen homogenates in triplicates onto Middlebrook 7H10 plates and incubated at 37°C for 15-17 days before enumeration. Balb/c mice were immunized intranasally (i.n.) with $5x10^7$ PFU of a replication-deficient human adenovirus-based TB vaccine (AdHuAg95A).

### *In vivo* T cell depletion

At 28 or 56 days post vaccination, mice were injected intraperitoneally (i.p.) with 200 µg of anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) or an IgG isotype control from Sigma-Aldrich (St. Louis, MO, USA). A second 100 µg dose was administered i.p. 2 days after.

Depletion was verified using flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA).

**Mononuclear cell isolation and purification**

The lungs were digested as described (41, 63, 64). The lungs were cut into small pieces and digested with 150U of collagenase type I from Gibco (Grand Island, NY, USA) in Hanks Buffer for 1 hr at 37˚C with agitation. The digested lungs were then crushed through a 100-µm filter from BD Falcon (Franklin Lakes, NJ, USA). Lungs were treated with ACK Lysis buffer for 2 minutes to remove all red blood cells. Cells were then resuspended in RPMI supplemented with 10% FBS, 1% Pen-Strep and 1% L-Glutamine (cRPMI). Isolated cells were resuspended in cRPMI and counted using Scepter cell counter from Millipore (Etobicoke, ON, CA).

The purification of $CD11c^+$ cells were performed as previously described (*16*) using MS column on the OctoMax separator according to manufacturer's instructions from Miltenyi Biotec (Auburn, CA, USA). Purity was assessed using flow cytometry on the LSRII using FACSDiva Software from BD Biosciences (San Jose, CA, USA).

**Cell surface and intracellular cytokine immunostaining**

T cell responses were analyzed using mononuclear cells isolated from the lung or lymph nodes and cultured in a 96 well plate at a concentration of $20x10^6$ cells/mL as previously described (41, 64, 65). For intracellular cytokine staining, cells were stimulated with 2µg of crude BCG and *M.tb* culture filtrate proteins for 24 hours. At the 18 hours, Golgi plug (5 µg/ml brefeldin A BD Bioscience, Burlington, ON, CA) was added to the culture at a concentration of 5µgl/mL. After incubation, cells were stained with blocked with CD16/CD32 for 15 minutes at 4°C, and stained with cell surface antibodies. In some cases, cells were washed and permeablized with Cytofix/cytoperm (BD Bioscience, Burlington, ON, CA), after which cells were stained

with intracellular cytokine antibodies. The following antibodies from BD Biosciences (San Jose, CA, USA) were used: CD3-V450, CD4-PE-Cy7, CD8a-APC-Cy7, IFN-γ-APC.

Immunoprofiling of innate immune cells were also performed by staining for cell surface markers as described using the following antibodies: CD45-APC-Cy7, CD11b-PE-Cy7, CD11c-APC from BD Biosciences (San Jose, CA, USA); MHC-II (I-A/I-E)-Alexa Fluor 700 and CD103-Biotin from eBiosciences (San Diego, CA, USA); Ly6C/G-Pacific Orange and Qdot800 from Invitrogen (Burlington, ON, Canada). All flow cytometry data was collected using the LSRII and FACSDiva software from BD Biosciences (San Jose, CA, USA) and analyzed using FlowJo Software from Treestar (Ashland, OR, USA).

**Mycobacterial killing assay**

Cells were isolated from naïve or i.n. AdHuAg85A immunized mice at 4 weeks post vaccination, after which $CD11c^+/CD11b^+$ cells were isolated from the lungs as described. Cells were plated at a density of $1x10^5$ cells per well in a 24 well plate, after which the cells were infected with *M.tb*H37Rv at a 1:1 ratio at 37°C. 4 hours post infection extracellular bacteria were removed, after which bacterial burden was assessed at 24 hour and 48 hour using a CFU assay.

**Cytokine and chemokine measurement**

Cytokine and chemokine levels within lung homogenates were determined using IFN-γ and TNF-α duo-set ELISA kit from R&D Systems (Burlington, ON, Canada).

**Statistical analysis**

Asterisks in the figures indicate the level of statistical significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)) as determined using a two-tailed student's T-test, One-Way ANOVA or Two-way ANOVA with a Tukey post-hoc analysis. Tests were performed using

GraphPad Prism software (La Jolla, CA, USA). Data are expressed as mean±SD unless otherwise stated.

## References


1. WHO, Global Tuberculosis Report 2016. (2016).
2. S. H. Kaufmann, Tuberculosis vaccines: time to think about the next generation. *Semin Immunol* **25**, 172-181 (2013).
3. Z. Xing, M. Jeyanathan, F. Smaill, New approaches to TB vaccination. *Chest* **146**, 804-812 (2014).
4. F. Smaill, M. Jeyanathan, M. Smieja, M. F. Medina, N. Thanthrige-Don, A. Zganiacz, C. Yin, A. Heriazon, D. Damjanovic, L. Puri, J. Hamid, F. Xie, R. Foley, J. Bramson, J. Gauldie, Z. Xing, A human type 5 adenovirus-based tuberculosis vaccine induces robust T cell responses in humans despite preexisting anti-adenovirus immunity. *Sci Transl Med* **5**, 205ra134 (2013).
5. J. Wang, L. Thorson, R. W. Stokes, M. Santosuosso, K. Huygen, A. Zganiacz, M. Hitt, Z. Xing, Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol* **173**, 6357-6365 (2004).
6. R. Lai, S. Afkhami, S. Haddadi, M. Jeyanathan, Z. Xing, Mucosal immunity and novel tuberculosis vaccine strategies: route of immunisation-determined T-cell homing to restricted lung mucosal compartments. *European respiratory review* **24**, 356-360 (2015).
7. V. N. Houk, J. H. Baker, K. Sorensen, D. C. Kent, The epidemiology of tuberculosis infection in a closed environment. *Arch Environ Health* **16**, 26-35 (1968).
8. T. Hussell, T. J. Bell, Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol* **14**, 81-93 (2014).
9. J. Pieters, Mycobacterium tuberculosis and the macrophage: maintaining a balance. *Cell Host Microbe* **3**, 399-407 (2008).
10. M. Jeyanathan, D. Damjanovic, C. R. Shaler, R. Lai, M. Wortzman, C. Yin, A. Zganiacz, B. D. Lichty, Z. Xing, Differentially imprinted innate immunity by mucosal boost vaccination determines antituberculosis immune protective outcomes, independent of T-cell immunity. *Mucosal Immunol* **6**, 612-625 (2013).
11. A. M. Cooper, Cell-Mediated Immune Responses in Tuberculosis. *Annual Review of Immunology* **27**, 393-422 (2009).
12. J. Wang, M. Santosuosso, P. Ngai, A. Zganiacz, Z. Xing, Activation of CD8 T cells by mycobacterial vaccination protects against pulmonary tuberculosis in the absence of CD4 T cells. *J Immunol* **173**, 4590-4597 (2004).
13. N. Tatsis, H. C. Ertl, Adenoviruses as vaccine vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* **10**, 616-629 (2004).
14. D. A. Muruve, The innate immune response to adenovirus vectors. *Human gene therapy* **15**, 1157-1166 (2004).
15. M. Santosuosso, S. McCormick, E. Roediger, X. Zhang, A. Zganiacz, B. D. Lichty, Z. Xing, Mucosal luminal manipulation of T cell geography switches on protective efficacy by otherwise ineffective parenteral genetic immunization. *J Immunol* **178**, 2387-2395 (2007).
16. C. N. Horvath, C. R. Shaler, M. Jeyanathan, A. Zganiacz, Z. Xing, Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: A critical role of airway luminal T cells. *Mucosal Immunol* **5**, 420-431 (2012).
17. R. Lai, M. Jeyanathan, C. R. Shaler, D. Damjanovic, A. Khera, C. Horvath, A. A. Ashkar, Z. Xing, Restoration of innate immune activation accelerates Th1 cell priming and protection following pulmonary mycobacterial infection. *Eur J Immunol*, (2014). **44**, 1375-86

18. J. Kleinnijenhuis, J. Quintin, F. Preijers, L. A. Joosten, C. Jacobs, R. J. Xavier, J. W. van der Meer, R. van Crevel, M. G. Netea, BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol* **155**, 213-219 (2014).
19. C. Naeslund, Experience de vaccination par le BCG dans la province de Norrbotten (Suede). *Revue de la Tuberculose* **12**, 617-636 (1931).
20. J. Chan, Y. Xing, R. S. Magliozzo, B. R. Bloom, Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* **175**, 1111-1122 (1992).
21. J. A. Philips, J. D. Ernst, Tuberculosis pathogenesis and immunity. *Annu Rev Pathol* **7**, 353-384 (2012).
22. K. N. Couper, D. G. Blount, J. C. Hafalla, N. van Rooijen, J. B. de Souza, E. M. Riley, Macrophage-mediated but gamma interferon-independent innate immune responses control the primary wave of Plasmodium yoelii parasitemia. *Infect Immun* **75**, 5806-5818 (2007).
23. M. Jeyanathan, N. Thanthrige-Don, S. Afkhami, R. Lai, D. Damjanovic, A. Zganiacz, X. Feng, X. D. Yao, K. L. Rosenthal, M. F. Medina, J. Gauldie, H. C. Ertl, Z. Xing, Novel chimpanzee adenovirus-vectored respiratory mucosal tuberculosis vaccine: overcoming local anti-human adenovirus immunity for potent TB protection. *Mucosal Immunol* **8**, 1373-1387 (2015).

**Figures**

**Figure 1**

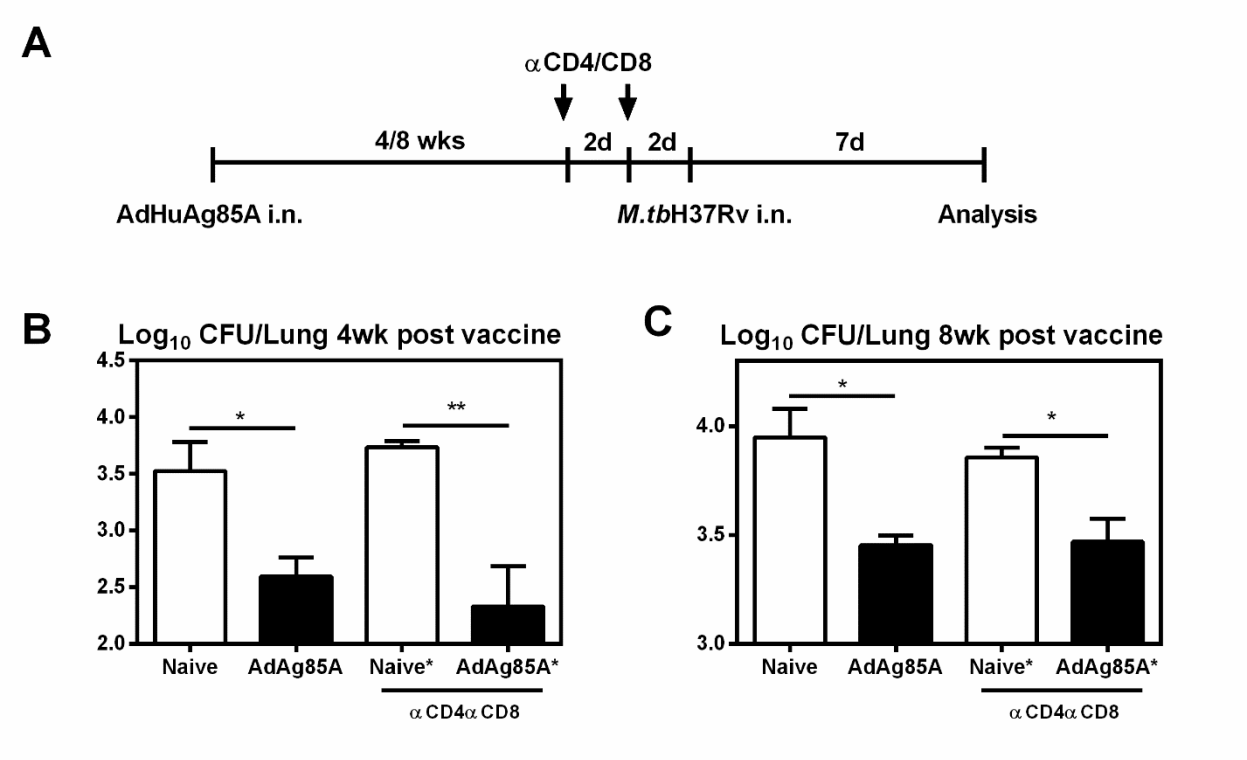


**Figure 2**

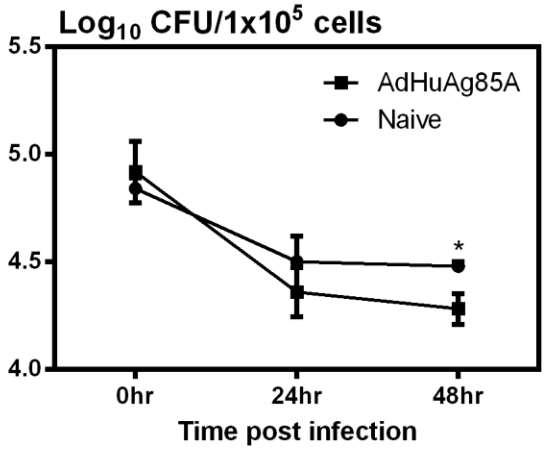

Log10 CFU/1x10^5 cells
5.5
5.0
4.5
4.0
AdHuAg85A
Naive
*
0hr
24hr
48hr
Time post infection

**Figure 3**

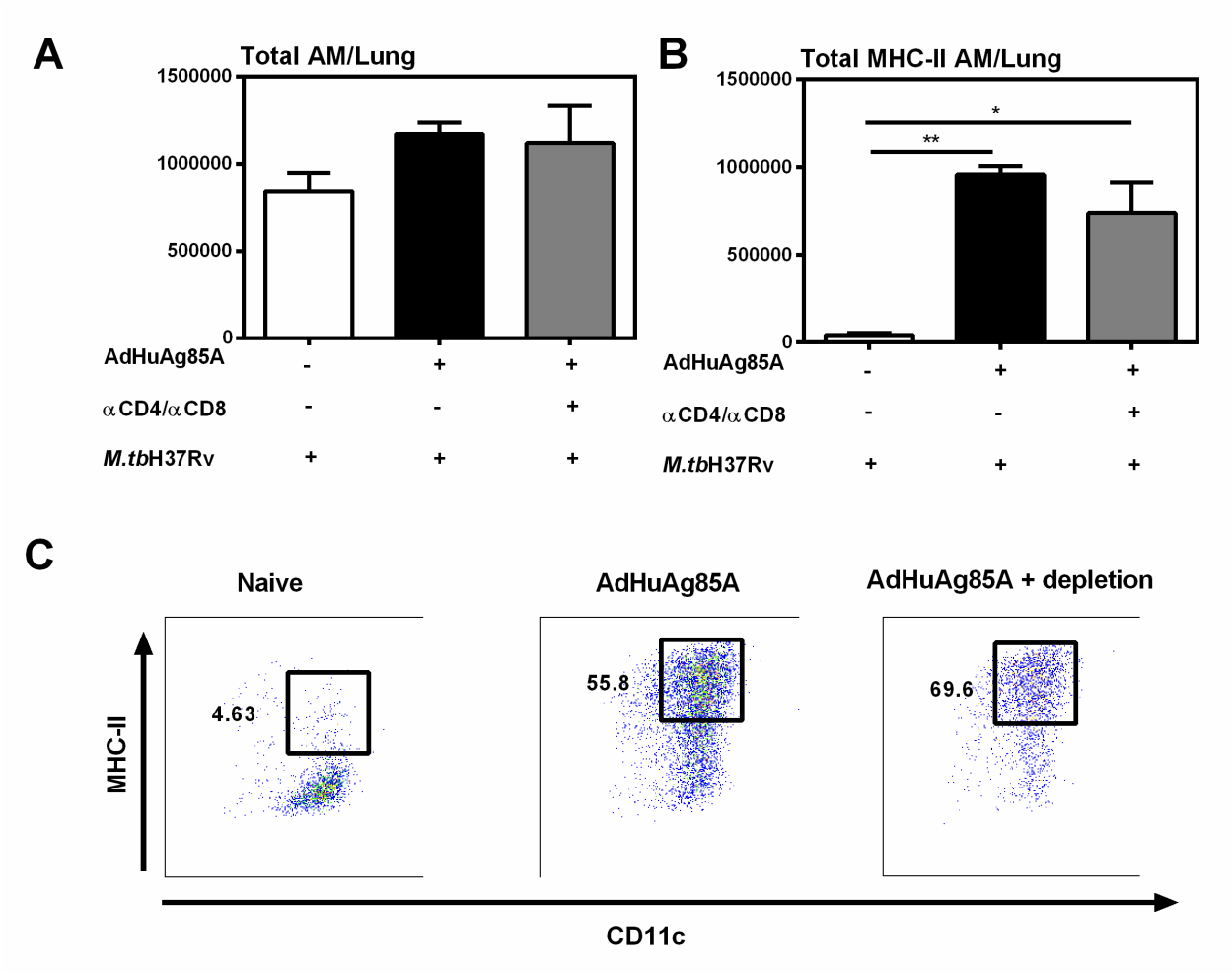

A
Total AM/Lung
1500000
1000000
500000
0
AdHuAg85A - + +
αCD4/αCD8 - - +
M.tbH37Rv + + +
B
Total MHC-II AM/Lung
1500000
1000000
500000
0
*
**
AdHuAg85A - + +
αCD4/αCD8 - - +
M.tbH37Rv + + +
C
Naive
AdHuAg85A
AdHuAg85A + depletion
4.63
55.8
69.6
MHC-II
CD11c

**Figure 4**

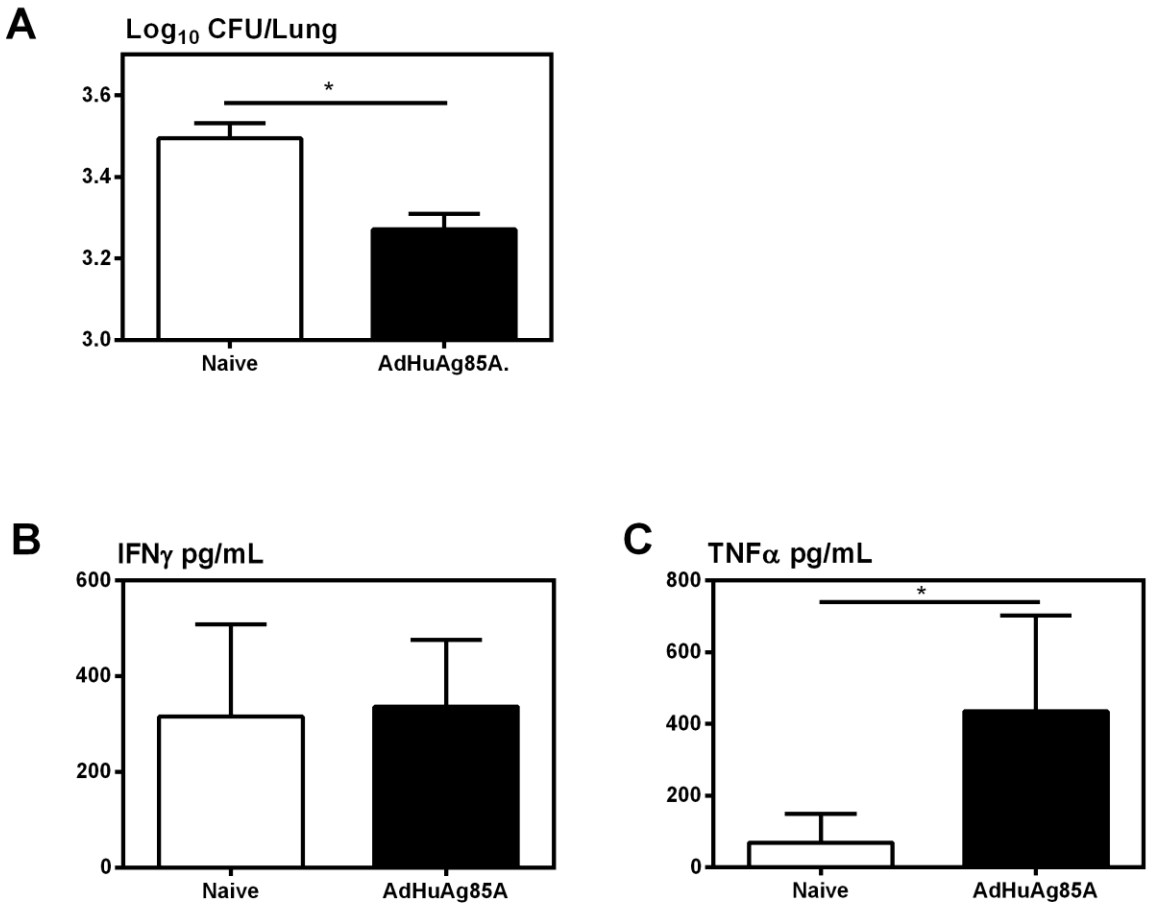

A
Log10 CFU/Lung
3.6
3.4
3.2
3.0
*
Naive
AdHuAg85A.
B
IFNγ pg/mL
600
400
200
0
Naive
AdHuAg85A
C
TNFα pg/mL
800
600
400
200
0
*
Naive
AdHuAg85A

**Figure S1**

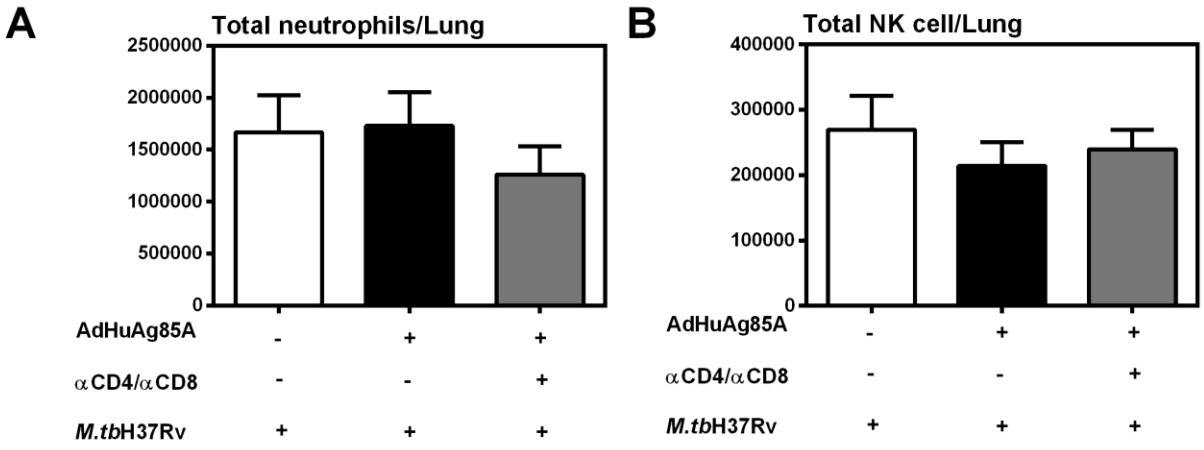

A
Total neutrophils/Lung
2500000
2000000
1500000
1000000
500000
0
AdHuAg85A - + +
αCD4/αCD8 - - +
M.tbH37Rv + + +
B
Total NK cell/Lung
400000
300000
200000
100000
0
AdHuAg85A - + +
αCD4/αCD8 - - +
M.tbH37Rv + + +

**Figure S2**

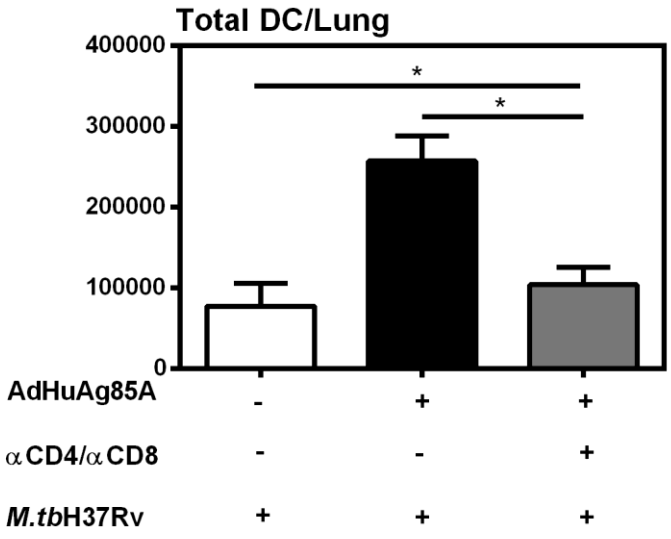

Total DC/Lung
400000
300000
200000
100000
0
*
*
AdHuAg85A
αCD4/αCD8
M.tbH37Rv
- + +
- - +
+ + +

**Table 1**

| Cell type | Markers |
|---|---|
| **Alveolar Macrophages (AM)** | **$CD45^{+}Gr\text{-}1^{-}DX5^{-}AF^{+}CD11c^{+}$ $MHC\text{-}II^{+/-}$** |
| **Dendritic cell (DC)** | **$CD45^{+}Gr\text{-}1^{-}DX5^{-}AF^{-}CD11c^{+}MHC\text{-}II^{+}$** |
| **Neutrophils** | **$CD45^{+}Gr\text{-}1^{+}CD11b^{+}$** |
| **NK cells** | **$CD45^{+}Gr\text{-}1^{-}DX5^{+}CD11b^{+}$** |

**Figure 1** – **Respiratory mucosal vaccination protects mice from pulmonary *M.tb* challenge in the absence of T cells for at least 7 days**. (A) Experimental schema describing how the experiment was carried out. Mice were immunized i.n. with AdHuAg85A for either (B) 4 weeks or (C) 8 weeks. In some groups T cells were depleted, after which mice were challenged with *M.tb*H37Rv. Bacterial burden was assessed 7 days post infection in the lung. Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

**Figure 2 – Lung innate cells exposed to AdHuAg85A have enhanced bactericidal abilities against *M.tb*.** Mice were immunized with AdHuAg85A for 4 weeks as described, after which $CD11c^+/CD11b^+$ cells were isolated from the lung using MACS purification. Cells were then infected with *M.tb* at a 1:1 ratio. After 4 hours, extracellular bacteria were removed, and bacterial burden in the cells were assessed at 0, 24 and 48 hours post infection. Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

**Figure 3** – **Alveolar macrophages have an activated phenotype following respiratory mucosal vaccination with AdHuAg85A, regardless of the presence of T cells**. Mice were immunized i.n. with AdHuAg85A, after which select groups were either depleted for T cells, infected with *M.tb* or both. Mononuclear cells were isolated from the lung at days 7 post infection, and the (A) total number of AM and (B) total number of MHC-$II^+$ AM were enumerated using flow cytometry. (C) Representative dot plot demonstrating the relative MHC-II expression on AM in each group. Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

**Figure 4** – **Respiratory mucosal vaccination protects mice from pulmonary *M.tb* challenge in SCID mice for at least 7 days**. SCID mice were immunized i.n. with AdHuAg85A for 4

weeks, after which mice were challenged with *M.tb*H37Rv. (A) Bacterial burden was assessed 7 days post infection in the lung. The levels of (B) IFNγ and (C) TNF-α were also assessed in the lung homogenates at this timepoint using ELISA. Data are expressed as mean±SD of three mice/time/group.

**Figure S1 – Number of neutrophils and NK cells following respiratory mucosal vaccination with AdHuAg85A.** Mice were immunized i.n. with AdHuAg85A, after which select groups were either depleted for T cells, infected with *M.tb* or both. Mononuclear cells were isolated from the lung at days 7 post infection, and the (A) total number of neutrophils and (B) NK cells were enumerated using flow cytometry. Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

**Figure S2 – Number of DC following respiratory mucosal vaccination with AdHuAg85A.** Mice were immunized i.n. with AdHuAg85A, after which select groups were either depleted for T cells, infected with *M.tb* or both. Mononuclear cells were isolated from the lung at days 7 post infection, and the total number of DC enumerated using flow cytometry. Data are expressed as mean±SD of three mice/time/group, representative of 2 independent experiments.

# Chapter 6. Discussion

## 6.1. Contributions to the field

Currently, BCG is the only clinically approved vaccine against TB. However, the substantial global burden of TB despite the use of BCG speaks to the ineffectiveness of current strategies used to control or eliminate the pathogen. Although multiple vaccine platforms are currently in development for the control of TB (*9*), our lack of understanding towards the correlates of protection against *M.tb* has hindered our progress in this regard (*9, 150*). Traditional strategies have involved targeting specific immunodominant epitopes expressed by *M.tb* to generate a robust Th1 immune response (*9, 151*). However, often ignored in the consideration of vaccine efficacy is the role of the innate immune compartment in host immunity against *M.tb*. The innate immune compartment serves as a critical aspect of anti-TB host immunity both in its capacity as the inducer of downstream adaptive T cell responses as well as being the primary effector population responsible for direct bacteria killing. Furthermore, given the ample amount of literature demonstrating the ability of *M.tb* to inhibit the functionality of the innate compartment (*28, 36, 78*), this becomes a critical area of research.

In **Chapter 3**, we have demonstrated that following pulmonary *M.tb* infection there is a substantial delay in the induction of early innate inflammatory factors such as TNF-α and IL-12. This lack of early inflammatory signals results in a delay in downstream immune responses including the migration of antigen-bearing DC as well as the activation of antigen-specific T cell responses. Furthermore, we demonstrated that enhancement of that early inflammatory response using a TLR4 agonist FimH led to an acceleration of downstream immune cascade, resulting in an earlier arrival of antigen-specific T cells at the site of infection in the lung and enhanced bacterial control. These findings demonstrate the importance of early innate inflammatory

responses in the generation of anti-TB Th1 immunity, and suggest that modulation of this compartment may be a viable strategy to enhance the ability of the host to generate anti-TB Th1 immunity.

In **Chapter 4**, we delved further into the mechanisms behind the generation and delay of anti-Th1 immunity. Specifically, we sought to understand the relative contribution of two recently identified migratory DC subsets, $CD11b^{+}$cDC and $CD103^{+}$cDC, in the delayed generation of Th1 immunity. We found that following pulmonary *M.tb* infection, both $CD11b^{+}$cDC and $CD103^{+}$cDC migrated to the dLN and were involved in the delivery of antigen to the dLN. However, it was $CD11b^{+}$cDC, but not $CD103^{+}$cDC, that was required for the generation of Th1 responses in the dLN. Instead, $CD103^{+}$cDC counter-regulated Th1 induction through the production of IL-10. These findings demonstrate a dichotomy in the functions of distinct DC populations upon pulmonary *M.tb* infection and identifies both the cell population responsible for Th1 activation and the one that may be involved in inhibiting it.

In **Chapters 3 and 4**, we established the importance of the innate immune compartment in the generation of anti-TB Th1 immunity following pulmonary *M.tb* infection. Previous work has identified that respiratory mucosal vaccination is able to provide superior protection against pulmonary *M.tb* infection in comparison to parenteral vaccination. While this is generally attributed to the presence of antigen-specific T cells in the lung and airway (*134*), it is unclear whether the innate immune compartment is also similarly modulated following respiratory mucosal vaccination. As such, in **Chapter 5**, we wanted to examine the relative contribution of both the innate and adaptive immune compartment in mediating anti-TB killing following respiratory mucosal vaccination with AdHuAg85A. We found that following respiratory mucosal vaccination with AdHuAg85A, mice were protected against *M.tb* during the first 7 days post

infection even in the absence of T cells. Furthermore, this was attributed to an enhanced activation in the lung AM population in AdHuAg85A vaccinated mice, leading to an enhanced mycobacterial killing capacity of these cells. Finally, we provided evidence demonstrating that T cells were not required to imprint the local lung innate cells as SCID mice were similarly protected following i.n. vaccination with AdHuAg85A. These findings demonstrate that in addition to inducing an antigen-specific T cell population at the lung, respiratory mucosal vaccination also modulates the lung local innate immune population independently of T cells and enhances its anti-TB functionality independently of T cell responses.

6.2. Modulation of innate immunity can lead to the acceleration of T cell priming

Our results from **chapter 3** have identified the early inflammatory response following pulmonary *M.tb* infection as a mechanism for delayed T cell activation. *M.tb* has been known to suppress many aspects of cellular function involved in T cell activation including the downregulation of MHC-II as well as antigen processing pathways (*50, 51, 152*). Furthermore, it has been demonstrated in an *ex vivo* setting that *M.tb* is able to suppress the secretion of inflammatory cytokines such as TNF-α and MCP-1 (*54, 55*). However, we are the first to examine the inflammatory responses *in vivo* during the early stages of pulmonary *M.tb* infection. A 5-10 day delay in the initiation of the inflammatory cascade would explain the characteristic 2 week delay in T cell priming at the draining lymph node following pulmonary *M.tb* infection, as the timing of T cell priming would be dependent on the initiation of the early inflammatory response.

However, rather than a lack of inflammatory responses some groups have previously attributed delayed T cell priming to a lack of sufficient antigenic load during the early phase of *M.tb* infection (*61, 63*), which in turn is attributed to the virulence of *M.tb* itself (*153*). This is

based on the observation that while cells infected with attenuated *M.tb*H37Ra undergo apoptotic cell death pathways, virulent *M.tb*H37Rv infection tends to promote necrotic cell death, thus preventing the efficient generation of *M.tb* antigens (*67, 68*). However, given that we have observe similar kinetics following both *M.tb*H37Ra and *M.tb*H37Rv infection (**chapter 3 and 4**), this is likely not the explanation for delayed T cell priming.

Instead, delayed T cell priming may be attributed to the ability of *M.tb*, or rather the entire *M.tb* family of mycobacterial subspecies, to suppress the early innate inflammatory response. Slow growing mycobacterial species, which include various *M.tb* strains as well as the BCG strain *M. bovis*, display the characteristic delay in T cell activation and persist for a long time within the host. On the other hand, fast growing mycobacterial species such as *M. smegmatis* or *M. abscessus* evoke a fast T cell response and are rapidly cleared (*154, 155*). One key difference between these two types of mycobacteria is the difference in the capping of the lipoarabinomannan (LAM) on the cell wall of the bacterium. Specifically, slow growing mycobacterial species have a mannose cap (manLAM) in comparison to the phosphor-*myo*-inositol (PI) capped LAM of the fast-growing species (*156, 157*). Interestingly, manLAM has been demonstrated to be highly immunosuppressive (*154*), and recently it has been demonstrated that it is able to induce the production of soluble TNF receptor without the production of TNF-α itself (*158*), thereby blocking immune activation and suggesting that this may be a mechanism by which *M.tb* inhibits early inflammatory responses.

As such, one goal of anti-TB vaccine design revolves around the acceleration of T cell kinetics in order to enhance Th1 responses in the lung. Our findings described in **chapter 3** have indicated that the use of the TLR4 agonist FimH may be a viable strategy to enhance early innate inflammatory responses following *M.tb* infection, as it has been demonstrated to enhance early

innate inflammatory responses and accelerate T cell immunity. Although the use of TLR agonists as an immune adjuvant is not a new concept, many are associated with significant side-effects or toxicity (*159, 160*). In contrast, FimH has been demonstrated to be a safe and effective immune modulator, and is particularly amenable for mucosal application as it is safe and induces minimum lung pathology (*161, 162*). FimH may be useful for accelerating T cell activation as we have observed accelerated T cell kinetics following FimH administration, which is also correlated with enhanced recruitment of APC to the lung.

6.3. Conventional DC subsets have divergent roles in Th1 activation following pulmonary *M.tb* infection

Our results from **chapter 3** also demonstrated that the administratiopn of FimH lead to the enhanced migration of lung DC. Given the central role for DC in T cell activation, the focus of **chapter 4** was to investigate the role of lung DC in Th1 activation following pulmonary *M.tb* infection. Although classically defined as simply being $CD11c^{+}MHC\text{-}II^{+}$ cells, recent efforts have refined the definition of DC, and have since identified different subsets within the previous umbrella of DC. Within the lung, two major populations, consisting of $CD11b^{+}$cDC and $CD103^{+}$cDC, have been shown to play non-redundant roles in orchestrating specific T cell activation in various models (*111, 112, 120, 163-165*). However, little is known about the role of these specifics DC subsets following pulmonary *M.tb* infection. We elected to focus on $CD11b^{+}$cDC and $CD103^{+}$cDC as these DC subsets are by far the best characterized and understood in literature, and have been demonstrated to be instrumental towards antigen presentation and T cell priming (*89, 96*).

Although a previous study had alluded to a role for $CD11b^{+}$cDC in the delivery of *M.tb* antigens to the dLN (*69*), our study demonstrates both DC subsets play a role in the delivery of

*M.tb* antigens to the dLN. Both DC subsets have previously demonstrated an ability to activate both $CD4^+$ and $CD8^+$ T cells in different settings. To date, $CD11b^+$cDC have been found to be critical primarily in Th2 and Th17 activation in models of allergy and extracellular parasite or fungal infection (*112, 163, 164, 166-168*), whereas $CD103^+$cDC have been found to initiate anti-intracellular parasitic Th1 activation (*165, 169*) or anti-viral $CD8^+$ T cell responses (*111, 114, 115*). Whether the preferential priming target of each DC subset is due to inherent differences in each DC subset is unclear. However, transcriptionally they are very distinct as $CD11b^+$cDC are dependent on IRF4 for their final differentiation, whereas $CD103^+$cDC are dependent on IRF8 and Batf3 (*106, 169, 170*). Furthermore, a gene analysis of each DC subset has observed a clustering of MHC-II associated genes around IRF4, whereas MHC-I associated genes clustered around IRF8 (*171*). Together, this suggests that each cDC lineage may specialize towards the activation of a specific T cell subset. However, $CD11b^+$cDC have also been found to prime and activate $CD8^+$ T cells (*113*). Furthermore, during RSV infection both DC subsets present antigen equally well (*116*). Altogether, the divergent roles of $CD11b^+$cDC and $CD103^+$cDC observed in literature suggest that the relative ability to activate $CD4^+$ or $CD8^+$ T cells by either DC subset is highly contextual and therefore cannot be generalized.

Interestingly the role of either DC subset in T cell activation has never been fully characterized following an intracellular bacterial infection. Using pulmonary *M.tb* in our model, we have found that $CD11b^+$cDC are the primary cell population responsible for the activation of Th1 cells, which is further confirmed by using $IRF4^{-/-}$ animals that are deficient in $CD11b^+$cDC. In contrast, we have observed a more regulatory role for $CD103^+$cDC following pulmonary *M.tb* infection. Certain immunoregulatory activities of $CD103^+$cDC have been demonstrated to be important for the induction of Tregs (*117, 118, 172*). However, our study has revealed a more

direct mechanism for CD103$^+$cDC in the inhibition of Th1 activation through the secretion of IL-10. While IL-10 has been implicated in the suppression of Th1 responses following *M.tb*, the cellular source within the dLN has never been previously defined. As such, our study provides a link between IL-10 and suppression of Th1 activation by identifying CD103$^+$cDC as a cellular source in the dLN during T cell priming.

In addition to playing a role in T cell activation, DC may also act as a natural reservoir for *M.tb*, as DC are in general unable to kill phagocytosed *M.tb* bacilli (*173, 174*). Their inability to kill *M.tb* makes them a perfect target for manipulation by the bacteria. Upon pulmonary *M.tb* infection, we find that CD103$^+$cDC have a higher bacterial burden at the dLN in comparison to CD103$^+$cDC (**chapter 4**). A similar increased susceptibility of CD103$^+$cDC was observed in a model of influenza infection (*175*). This may highlight an increased susceptibility of CD103$^+$cDC to the entry of *M.tb* in comparison to CD11b$^+$cDC. On the other hand, this may simply be attributed to the anatomical location of CD103$^+$cDC as these cells are found within the epithelial layer (*176*) and would therefore have greater access to pathogens within the airway than CD11b$^+$cDC. The increased production of IL-10 by CD103$^+$cDC would lead to the suppression of processes involved in bacterial killing such as the production of reactive oxygen and nitrogen species (*73, 177*), and may explain why DC have been found unable to kill *M.tb* in the past. However, the difference in IL-10 production between CD103$^+$cDC and CD11b$^+$cDC may also reflect a difference in bactericidal capabilities between these two DC subsets.

Together, our results from **chapter 3** and **chapter 4** provide a target for manipulation to enhance and accelerate Th1 immunity, and provide us with further rationale to use innate modulation as a means by which we can accelerate Th1 immunity. Given the production of IL-10 by CD103$^+$cDC, it is tempting to suggest that blockade or removal of this population may be a

means to accelerating Th1 immunity. However, it has been demonstrated that $CD103^+$cDC are critical in the production of IL-12 (*169, 178*), which is required for the polarization of Th1 cells. As such, the removal of this cell population may instead further impair the generation of Th1 responses. Instead, an alternative strategy for accelerating T cell activation may be the use of FimH, as we have already demonstrated its role in accelerating the migration of DC to the dLN (**chapter 3**). Interestingly, we have also demonstrated that the administration of FimH lead to a specific acceleration of $CD11b^+$cDC migration but not $CD103^+$cDC (unpublished data). Given that in **chapter 4** we have demonstrated the critical role for $CD11b^+$cDC in Th1 priming, the use of FimH may allow us to specifically manipulate this key DC population needed for Th1 activation. However, further study would be required to identify the specific mechanism by which FimH specifically accelerates the migration of $CD11b^+$cDC.

6.4. The innate compartment contributes to host defense against *M.tb* independent of T cell responses

Our results from **chapter 3** and **chapter 4** highlight a potential target of manipulation for accelerating Th1 immunity, and provide us with a strategy by which this may be achieved. The success of BCG is often linked with its ability to accelerate Th1 immunity by at least one week (*7, 18*), However, despite this acceleration in T cell responses BCG efficacy wanes over time and is unable to protect against pulmonary TB. This suggests that the acceleration of T cell responses alone is not sufficient to mediate protective immunity against pulmonary TB. As such, modern vaccination strategies have sought to build upon the success of BCG and now seek to further accelerate or enhance the quality of Th1 immunity as a means to enhance protection against pulmonary *M.tb (7, 9)*.

Given that BCG is delivered parenterally, it is likely that the vaccine is unable to directly modulate the lung compartment. This is likely one reason for the failure of BCG to mediate long lasting protection against pulmonary *M.tb*, as we and others have demonstrated the superiority of mucosal vaccination over parenteral vaccination in the induction of long term memory responses (*134, 137, 179*). Our lab has demonstrated that the use of an adenovirus serotype 5 based mucosal vaccine expressing an *M.tb* immunodominant protein Ag85A (hereby referred to as AdHuAg85A) is particularly effective in providing protection against *M.tb*. Although our clinical data and animal models have demonstrated that it is particularly effective in generating long lasting IFN-$\gamma^+$ TNF-$\alpha^+$ $CD8^+$ T cells, and to a lesser extent $CD4^+$ T-cell responses (*129, 134, 135, 180-182*), our preclinical data has demonstrated the effectiveness of AdHuAg85A can be further enhanced if delivered mucosally. We have demonstrated that respiratory mucosal vaccination with AdHuAg85A is able to induce a population of Ag-specific T cells in the airway and the lung and superior protection over traditional parenteral vaccination. However, the immunogenic nature of adenoviral vectors (*140, 141*) makes it likely that the local innate immune compartment is also similarly activated. Given that our data from **chapter 3** and **chapter 4** suggests targeting the innate immune compartment as a complementary approach to traditional vaccine platforms, we sought to address how respiratory mucosal vaccination with AdHuAg85A would modulate the local innate compartment.

Collectively, our results from **chapter 5** demonstrate the importance of the innate immune compartment in direct killing of *M.tb* independent of T cell responses following respiratory mucosal vaccination. Although previously it was thought that the enhanced protection observed is likely due to the specific presence of antigen-specific T cells within the lung tissue itself, our data demonstrates that this may also be due to an enhanced innate immune response, as

modulation of the innate compartment not only accelerated their antigen delivery and presentation capacity (**Chapter 3**), but also enhanced their bactericidal capabilities (**Chapter 5**). As such, when evaluating the efficacy of a mucosally administered vaccine, the effects a specific vaccine formulation has on the local innate immune compartment should also be considered. Our lab has also demonstrated that the effects of the vaccine platform has on the innate compartment can greatly impact the efficacy of given vaccine formulation even when it is able to induce a robust adaptive Th1 immunity (*143*).

The importance of the innate immune compartment is not only restricted to T cell priming or direct killing of *M.tb*. A recent study has demonstrated that lung DC were able to specifically recruit T cells back to the lung in comparison to DC from other compartments through the upregulation of a chemokine receptor CCR4 (*183*), demonstrating that the local innate immune cells are also critical in directing T cell responses to specific tissue compartments. Our lab and others have also identified other markers such as VLA-1, CCR5 and CXCR3 (*184, 185*). Although we have demonstrated an increase in chemokine receptors following respiratory mucosal vaccination with AdHuAg85A in comparison to parenteral vaccination (data not shown), it is not clear which DC subset, if any, is involved in conferring this imprinted phenotype. Our study has identified $CD11b^{+}$cDC as a potential DC subset responsible for recruiting antigen-specific T cells back to the lung, as these cells are critical to the induction of Th1 immunity at the dLN following *M.tb* infection (**Chapter 4**), and there appears to be an increased presence of $CD11b^{+}$cDC in the lung and lung dLN following respiratory mucosal vaccination with AdHuAg85A (data not shown). Further study is required to determine how intranasal AdHuAg85A vaccination modulates the lung DC population and whether this affects their ability to draw antigen-specific T cells to the lung.

Although traditional vaccine immunology has suggested that only the adaptive immunity is capable of memory responses, there is mounting evidence suggesting that the innate immune compartment can also be educated against a pathogen. Termed trained innate immunity, this concept revolves around the idea that exposure of the innate compartment can be influenced by previous encounters to pathogens or their byproducts, thereby modulating their response in comparison to an "uneducated" host innate immune system (*186*). The earliest evidence of this stems from a study of a TB outbreak on an US navel ship where they found 13 individuals who remained protected despite the absence of anti-TB T cell immunity for at least 6 months post exposure (*144*), suggesting that the protection was mediated by the host innate immunity independently of T cells. Since then, BCG has also been shown to modulate innate monocytes and NK cells through epigenetic changes that persist for at least 6 months (*147, 148*). However, as BCG is delivered parenterally, it is not clear whether it is able to modulate the lung local innate cells, and if so for how long. Given the enhanced activation seen in the innate immune cells following intranasal AdHuAg85A vaccination (**Chapter 5**), it is likely that mucosal delivery of a vaccine may allow for long term epigenetic modifications of the innate compartment.

### 6.5. The importance of examining the innate compartment in current and future clinical vaccine trials

The induction of Th1 immunity remains critical in anti-TB immunity, and as such will continue to be a central part of anti-TB vaccine design. Our work has demonstrated the importance of the innate compartment towards the generation of anti-TB Th1 immunity as well as the direct control of *M.tb*. As such, equal consideration must be given towards the innate immune compartment when evaluating the efficacy of a vaccine platform. We believe that the ability of respiratory mucosal vaccination to manipulate the local lung innate immune

compartment makes it a superior route of vaccination than traditional parenteral vaccination strategies. Currently, we are in the middle of an effort to examine the efficacy of respiratory AdHuAg85A delivery in BCG immunized human volunteers. However, as a result of the work encompassed by this thesis, we will also be evaluating the innate immune responses in addition to traditional Ag-specific T cell responses in these volunteers. This new information will provide us with more tools to evaluate and predict the protective efficacy of our vaccine formulation and delivery strategy. However, we believe in the necessity of evaluating the innate immune compartment in all future vaccine trials irrespective of the formulation or the route of delivery, as this will provide us with more information towards generating protective immunity against any given disease.

# References


1. WHO, Global Tuberculosis Report 2016. (2016).
2. M. Lerm, M. G. Netea, Trained immunity: a new avenue for tuberculosis vaccine development. *J Intern Med* **279**, 337-346 (2016).
3. E. A. Miller, J. D. Ernst, Anti-TNF immunotherapy and tuberculosis reactivation: another mechanism revealed. *J Clin Invest* **119**, 1079-1082 (2009).
4. S. D. Lawn, A. I. Zumla, Tuberculosis. *Lancet* **378**, 57-72 (2011).
5. S. Luca, T. Mihaescu, History of BCG Vaccine. *Maedica (Buchar)* **8**, 53-58 (2013).
6. S. H. Kaufmann, Tuberculosis vaccines: time to think about the next generation. *Semin Immunol* **25**, 172-181 (2013).
7. P. Andersen, T. M. Doherty, The success and failure of BCG - implications for a novel tuberculosis vaccine. *Nat Rev Microbiol* **3**, 656-662 (2005).
8. I. Abubakar, L. Pimpin, C. Ariti, R. Beynon, P. Mangtani, J. A. Sterne, P. E. Fine, P. G. Smith, M. Lipman, D. Elliman, J. M. Watson, L. N. Drumright, P. F. Whiting, E. Vynnycky, L. C. Rodrigues, Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette-Guerin vaccination against tuberculosis. *Health Technol Assess* **17**, 1-372, v-vi (2013).
9. Z. Xing, M. Jeyanathan, F. Smaill, New approaches to TB vaccination. *Chest* **146**, 804-812 (2014).
10. C. E. Barry, 3rd, H. I. Boshoff, V. Dartois, T. Dick, S. Ehrt, J. Flynn, D. Schnappinger, R. J. Wilkinson, D. Young, The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* **7**, 845-855 (2009).
11. L. Ramakrishnan, Revisiting the role of the granuloma in tuberculosis. *Nat Rev Immunol* **12**, 352-366 (2012).
12. C. R. Shaler, C. N. Horvath, M. Jeyanathan, Z. Xing, Within the Enemy's Camp: contribution of the granuloma to the dissemination, persistence and transmission of Mycobacterium tuberculosis. *Front Immunol* **4**, 30 (2013).

13. D. R. Roach, A. G. Bean, C. Demangel, M. P. France, H. Briscoe, W. J. Britton, TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol* **168**, 4620-4627 (2002).
14. B. M. Saunders, W. J. Britton, Life and death in the granuloma: immunopathology of tuberculosis. *Immunol Cell Biol* **85**, 103-111 (2007).
15. M. J. Kim, H. C. Wainwright, M. Locketz, L. G. Bekker, G. B. Walther, C. Dittrich, A. Visser, W. Wang, F. F. Hsu, U. Wiehart, L. Tsenova, G. Kaplan, D. G. Russell, Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. *EMBO Mol Med* **2**, 258-274 (2010).
16. J. H. Chin, Tuberculous meningitis: Diagnostic and therapeutic challenges. *Neurol Clin Pract* **4**, 199-205 (2014).
17. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. *Am J Respir Crit Care Med* **161**, 1376-1395 (2000).
18. A. M. Cooper, Cell-Mediated Immune Responses in Tuberculosis. *Annual Review of Immunology* **27**, 393-422 (2009).
19. I. M. Orme, The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with Mycobacterium tuberculosis. *J Immunol* **138**, 293-298 (1987).
20. A. A. Chackerian, T. V. Perera, S. M. Behar, Gamma interferon-producing CD4+ T lymphocytes in the lung correlate with resistance to infection with Mycobacterium tuberculosis. *Infect Immun* **69**, 2666-2674 (2001).
21. W. A. Hanekom, The immune response to BCG vaccination of newborns. *Ann N Y Acad Sci* **1062**, 69-78 (2005).
22. S. A. Khader, G. K. Bell, J. E. Pearl, J. J. Fountain, J. Rangel-Moreno, G. E. Cilley, F. Shen, S. M. Eaton, S. L. Gaffen, S. L. Swain, R. M. Locksley, L. Haynes, T. D. Randall, A. M. Cooper, 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**, 369-377 (2007).
23. S. Sakai, K. D. Kauffman, M. A. Sallin, A. H. Sharpe, H. A. Young, V. V. Ganusov, D. L. Barber, CD4 T Cell-Derived IFN-gamma Plays a Minimal Role in Control of Pulmonary Mycobacterium tuberculosis Infection and Must Be Actively Repressed by PD-1 to Prevent Lethal Disease. *PLoS Pathog* **12**, e1005667 (2016).
24. J. L. Flynn, J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, B. R. Bloom, An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med* **178**, 2249-2254 (1993).
25. A. M. Cooper, D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, I. M. Orme, Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* **178**, 2243-2247 (1993).
26. N. Remus, J. Reichenbach, C. Picard, C. Rietschel, P. Wood, D. Lammas, D. S. Kumararatne, J. L. Casanova, Impaired interferon gamma-mediated immunity and susceptibility to mycobacterial infection in childhood. *Pediatr Res* **50**, 8-13 (2001).
27. J. L. Casanova, L. Abel, Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol* **20**, 581-620 (2002).
28. C. R. Shaler, C. Horvath, R. Lai, Z. Xing, Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clin Dev Immunol* **2012**, 628293 (2012).
29. E. Ingulli, C. Funatake, E. L. Jacovetty, M. Zanetti, Cutting edge: antigen presentation to CD8 T cells after influenza A virus infection. *J Immunol* **182**, 29-33 (2009).

30. L. A. Zenewicz, H. Shen, Innate and adaptive immune responses to Listeria monocytogenes: a short overview. *Microbes Infect* **9**, 1208-1215 (2007).
31. K. B. Urdahl, S. Shafiani, J. D. Ernst, Initiation and regulation of T-cell responses in tuberculosis. *Mucosal Immunol* **4**, 288-293 (2011).
32. T. Hussell, T. J. Bell, Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol* **14**, 81-93 (2014).
33. D. Bedoret, H. Wallemacq, T. Marichal, C. Desmet, F. Quesada Calvo, E. Henry, R. Closset, B. Dewals, C. Thielen, P. Gustin, L. de Leval, N. Van Rooijen, A. Le Moine, A. Vanderplasschen, D. Cataldo, P. V. Drion, M. Moser, P. Lekeux, F. Bureau, Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *J Clin Invest* **119**, 3723-3738 (2009).
34. M. Kopf, C. Schneider, S. P. Nobs, The development and function of lung-resident macrophages and dendritic cells. *Nat Immunol* **16**, 36-44 (2015).
35. P. R. Taylor, L. Martinez-Pomares, M. Stacey, H. H. Lin, G. D. Brown, S. Gordon, Macrophage receptors and immune recognition. *Annu Rev Immunol* **23**, 901-944 (2005).
36. J. A. Philips, J. D. Ernst, Tuberculosis pathogenesis and immunity. *Annu Rev Pathol* **7**, 353-384 (2012).
37. M. Guilliams, I. De Kleer, S. Henri, S. Post, L. Vanhoutte, S. De Prijck, K. Deswarte, B. Malissen, H. Hammad, B. N. Lambrecht, Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med* **210**, 1977-1992 (2013).
38. D. Hashimoto, A. Chow, C. Noizat, P. Teo, M. B. Beasley, M. Leboeuf, C. D. Becker, P. See, J. Price, D. Lucas, M. Greter, A. Mortha, S. W. Boyer, E. C. Forsberg, M. Tanaka, N. van Rooijen, A. Garcia-Sastre, E. R. Stanley, F. Ginhoux, P. S. Frenette, M. Merad, Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* **38**, 792-804 (2013).
39. S. Yona, K. W. Kim, Y. Wolf, A. Mildner, D. Varol, M. Breker, D. Strauss-Ayali, S. Viukov, M. Guilliams, A. Misharin, D. A. Hume, H. Perlman, B. Malissen, E. Zelzer, S. Jung, Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* **38**, 79-91 (2013).
40. U. A. Maus, S. Janzen, G. Wall, M. Srivastava, T. S. Blackwell, J. W. Christman, W. Seeger, T. Welte, J. Lohmeyer, Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. *Am J Respir Cell Mol Biol* **35**, 227-235 (2006).
41. C. Jakubzick, E. L. Gautier, S. L. Gibbings, D. K. Sojka, A. Schlitzer, T. E. Johnson, S. Ivanov, Q. Duan, S. Bala, T. Condon, N. van Rooijen, J. R. Grainger, Y. Belkaid, A. Ma'ayan, D. W. Riches, W. M. Yokoyama, F. Ginhoux, P. M. Henson, G. J. Randolph, Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* **39**, 599-610 (2013).
42. S. Tamoutounour, M. Guilliams, F. Montanana Sanchis, H. Liu, D. Terhorst, C. Malosse, E. Pollet, L. Ardouin, H. Luche, C. Sanchez, M. Dalod, B. Malissen, S. Henri, Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* **39**, 925-938 (2013).
43. S. Ensan, A. Li, R. Besla, N. Degousee, J. Cosme, M. Roufaiel, E. A. Shikatani, M. El-Maklizi, J. W. Williams, L. Robins, C. Li, B. Lewis, T. J. Yun, J. S. Lee, P. Wieghofer, R. Khattar, K. Farrokhi, J. Byrne, M. Ouzounian, C. C. Zavitz, G. A. Levy, C. M. Bauer, P. Libby, M. Husain, F. K. Swirski, C. Cheong, M. Prinz, I. Hilgendorf, G. J. Randolph, S. Epelman, A. O. Gramolini, M. I. Cybulsky, B. B. Rubin, C. S. Robbins, Self-renewing resident arterial macrophages arise from embryonic CX3CR1(+) precursors and circulating monocytes immediately after birth. *Nat Immunol* **17**, 159-168 (2016).

44. E. L. Gautier, T. Shay, J. Miller, M. Greter, C. Jakubzick, S. Ivanov, J. Helft, A. Chow, K. G. Elpek, S. Gordonov, A. R. Mazloom, A. Ma'ayan, W. J. Chua, T. H. Hansen, S. J. Turley, M. Merad, G. J. Randolph, C. Immunological Genome, Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* **13**, 1118-1128 (2012).
45. S. L. Gibbings, S. M. Thomas, S. M. Atif, A. L. McCubbrey, A. N. Desch, T. Danhorn, S. M. Leach, D. L. Bratton, P. M. Henson, W. J. Janssen, C. V. Jakubzick, Three Unique Interstitial Macrophages in the Murine Lung at Steady State. *Am J Respir Cell Mol Biol*, (2017).
46. Y. Cai, C. Sugimoto, M. Arainga, X. Alvarez, E. S. Didier, M. J. Kuroda, In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans. *J Immunol* **192**, 2821-2829 (2014).
47. A. Bafica, C. A. Scanga, C. G. Feng, C. Leifer, A. Cheever, A. Sher, TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J Exp Med* **202**, 1715-1724 (2005).
48. D. Sanchez, M. Rojas, I. Hernandez, D. Radzioch, L. F. Garcia, L. F. Barrera, Role of TLR2- and TLR4-mediated signaling in Mycobacterium tuberculosis-induced macrophage death. *Cell Immunol* **260**, 128-136 (2010).
49. M. Divangahi, S. Mostowy, F. Coulombe, R. Kozak, L. Guillot, F. Veyrier, K. S. Kobayashi, R. A. Flavell, P. Gros, M. A. Behr, NOD2-deficient mice have impaired resistance to Mycobacterium tuberculosis infection through defective innate and adaptive immunity. *J Immunol* **181**, 7157-7165 (2008).
50. N. D. Pecora, S. A. Fulton, S. M. Reba, M. G. Drage, D. P. Simmons, N. J. Urankar-Nagy, W. H. Boom, C. V. Harding, Mycobacterium bovis BCG decreases MHC-II expression in vivo on murine lung macrophages and dendritic cells during aerosol infection. *Cell Immunol* **254**, 94-104 (2009).
51. L. Ramachandra, D. Simmons, C. V. Harding, MHC molecules and microbial antigen processing in phagosomes. *Curr Opin Immunol* **21**, 98-104 (2009).
52. K. Rohde, R. M. Yates, G. E. Purdy, D. G. Russell, Mycobacterium tuberculosis and the environment within the phagosome. *Immunol Rev* **219**, 37-54 (2007).
53. D. L. Clemens, M. A. Horwitz, Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181**, 257-270 (1995).
54. M. Chieppa, G. Bianchi, A. Doni, A. Del Prete, M. Sironi, G. Laskarin, P. Monti, L. Piemonti, A. Biondi, A. Mantovani, M. Introna, P. Allavena, Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J Immunol* **171**, 4552-4560 (2003).
55. R. I. Tapping, P. S. Tobias, Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *J Endotoxin Res* **9**, 264-268 (2003).
56. B. N. Lambrecht, Allergen uptake and presentation by dendritic cells. *Curr Opin Allergy Clin Immunol* **1**, 51-59 (2001).
57. F. Sallusto, P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C. R. Mackay, S. Qin, A. Lanzavecchia, Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* **28**, 2760-2769 (1998).
58. S. A. Khader, S. Partida-Sanchez, G. Bell, D. M. Jelley-Gibbs, S. Swain, J. E. Pearl, N. Ghilardi, F. J. Desauvage, F. E. Lund, A. M. Cooper, Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection. *J Exp Med* **203**, 1805-1815 (2006).
59. J. L. Herrmann, P. H. Lagrange, Dendritic cells and Mycobacterium tuberculosis: which is the Trojan horse? *Pathol Biol (Paris)* **53**, 35-40 (2005).

60. M. Nagl, L. Kacani, B. Mullauer, E. M. Lemberger, H. Stoiber, G. M. Sprinzl, H. Schennach, M. P. Dierich, Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells. *Clin Diagn Lab Immunol* **9**, 1165-1168 (2002).
61. A. J. Wolf, L. Desvignes, B. Linas, N. Banaiee, T. Tamura, K. Takatsu, J. D. Ernst, Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. *J Exp Med* **205**, 105-115 (2008).
62. R. Lai, M. Jeyanathan, C. R. Shaler, D. Damjanovic, A. Khera, C. Horvath, A. A. Ashkar, Z. Xing, Restoration of innate immune activation accelerates Th1 cell priming and protection following pulmonary mycobacterial infection. *Eur J Immunol*, (2014).
63. M. S. Russell, M. Iskandar, O. L. Mykytczuk, J. H. Nash, L. Krishnan, S. Sad, A reduced antigen load in vivo, rather than weak inflammation, causes a substantial delay in CD8+ T cell priming against Mycobacterium bovis (bacillus Calmette-Guerin). *J Immunol* **179**, 211-220 (2007).
64. D. Ordway, M. Henao-Tamayo, E. Smith, C. Shanley, M. Harton, J. Troudt, X. Bai, R. J. Basaraba, I. M. Orme, E. D. Chan, Animal model of Mycobacterium abscessus lung infection. *J Leukoc Biol* **83**, 1502-1511 (2008).
65. M. Divangahi, M. Chen, H. Gan, D. Desjardins, T. T. Hickman, D. M. Lee, S. Fortune, S. M. Behar, H. G. Remold, Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. *Nat Immunol* **10**, 899-906 (2009).
66. U. E. Schaible, F. Winau, P. A. Sieling, K. Fischer, H. L. Collins, K. Hagens, R. L. Modlin, V. Brinkmann, S. H. Kaufmann, Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. *Nat Med* **9**, 1039-1046 (2003).
67. M. Divangahi, D. Desjardins, C. Nunes-Alves, H. G. Remold, S. M. Behar, Eicosanoid pathways regulate adaptive immunity to Mycobacterium tuberculosis. *Nat Immunol* **11**, 751-758 (2010).
68. R. Blomgran, L. Desvignes, V. Briken, J. D. Ernst, Mycobacterium tuberculosis inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host Microbe* **11**, 81-90 (2012).
69. A. J. Wolf, B. Linas, G. J. Trevejo-Nunez, E. Kincaid, T. Tamura, K. Takatsu, J. D. Ernst, Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. *J Immunol* **179**, 2509-2519 (2007).
70. L. Ramachandra, E. Noss, W. H. Boom, C. V. Harding, Processing of Mycobacterium tuberculosis antigen 85B involves intraphagosomal formation of peptide-major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. *J Exp Med* **194**, 1421-1432 (2001).
71. L. Tailleux, O. Schwartz, J. L. Herrmann, E. Pivert, M. Jackson, A. Amara, L. Legres, D. Dreher, L. P. Nicod, J. C. Gluckman, P. H. Lagrange, B. Gicquel, O. Neyrolles, DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. *J Exp Med* **197**, 121-127 (2003).
72. D. F. Fiorentino, A. Zlotnik, T. R. Mosmann, M. Howard, A. O'Garra, IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **147**, 3815-3822 (1991).
73. R. T. Gazzinelli, I. P. Oswald, S. L. James, A. Sher, IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. *J Immunol* **148**, 1792-1796 (1992).
74. K. W. Moore, R. de Waal Malefyt, R. L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* **19**, 683-765 (2001).
75. L. Williams, L. Bradley, A. Smith, B. Foxwell, Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* **172**, 567-576 (2004).
76. K. C. El Kasmi, J. Holst, M. Coffre, L. Mielke, A. de Pauw, N. Lhocine, A. M. Smith, R. Rutschman, D. Kaushal, Y. Shen, T. Suda, R. P. Donnelly, M. G. Myers, Jr., W. Alexander, D. A. Vignali, S. S. Watowich, M. Ernst, D. J. Hilton, P. J. Murray, General nature of the STAT3-activated anti-inflammatory response. *J Immunol* **177**, 7880-7888 (2006).

77. S. K. Pathak, S. Basu, A. Bhattacharyya, S. Pathak, M. Kundu, J. Basu, Mycobacterium tuberculosis lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. *J Biol Chem* **280**, 42794-42800 (2005).
78. P. S. Redford, P. J. Murray, A. O'Garra, The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunol* **4**, 261-270 (2011).
79. T. B. Geijtenbeek, S. J. Van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. Vandenbroucke-Grauls, B. Appelmelk, Y. Van Kooyk, Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* **197**, 7-17 (2003).
80. C. Demangel, P. Bertolino, W. J. Britton, 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).
81. M. Jeyanathan, S. McCormick, R. Lai, S. Afkhami, C. R. Shaler, C. N. Horvath, D. Damjanovic, A. Zganiacz, N. Barra, A. Ashkar, M. Jordana, N. Aoki, Z. Xing, Pulmonary M. tuberculosis infection delays Th1 immunity via immunoadaptor DAP12-regulated IRAK-M and IL-10 expression in antigen-presenting cells. *Mucosal Immunol*, (2013).
82. P. S. Redford, A. Boonstra, S. Read, J. Pitt, C. Graham, E. Stavropoulos, G. J. Bancroft, A. O'Garra, 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**, 2200-2210 (2010).
83. G. L. Beamer, D. K. Flaherty, B. D. Assogba, P. Stromberg, M. Gonzalez-Juarrero, R. de Waal Malefyt, B. Vesosky, J. Turner, Interleukin-10 promotes Mycobacterium tuberculosis disease progression in CBA/J mice. *J Immunol* **181**, 5545-5550 (2008).
84. C. Manca, L. Tsenova, A. Bergtold, S. Freeman, M. Tovey, J. M. Musser, C. E. Barry, 3rd, V. H. Freedman, G. Kaplan, Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta. *Proc Natl Acad Sci U S A* **98**, 5752-5757 (2001).
85. C. Manca, L. Tsenova, S. Freeman, A. K. Barczak, M. Tovey, P. J. Murray, C. Barry, G. Kaplan, Hypervirulent M. tuberculosis W/Beijing strains upregulate type I IFNs and increase expression of negative regulators of the Jak-Stat pathway. *J Interferon Cytokine Res* **25**, 694-701 (2005).
86. F. W. McNab, J. Ewbank, A. Howes, L. Moreira-Teixeira, A. Martirosyan, N. Ghilardi, M. Saraiva, A. O'Garra, Type I IFN induces IL-10 production in an IL-27-independent manner and blocks responsiveness to IFN-gamma for production of IL-12 and bacterial killing in Mycobacterium tuberculosis-infected macrophages. *J Immunol* **193**, 3600-3612 (2014).
87. B. Wojtas, B. Fijalkowska, A. Wlodarczyk, A. Schollenberger, M. Niemialtowski, B. Hamasur, A. Pawlowski, M. Krzyzowska, Mannosylated lipoarabinomannan balances apoptosis and inflammatory state in mycobacteria-infected and uninfected bystander macrophages. *Microb Pathog* **51**, 9-21 (2011).
88. R. M. Steinman, M. D. Witmer, Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* **75**, 5132-5136 (1978).
89. V. Durai, K. M. Murphy, Functions of Murine Dendritic Cells. *Immunity* **45**, 719-736 (2016).
90. D. Amsen, C. G. Spilianakis, R. A. Flavell, How are T(H)1 and T(H)2 effector cells made? *Curr Opin Immunol* **21**, 153-160 (2009).
91. A. T. Satpathy, X. Wu, J. C. Albring, K. M. Murphy, Re(de)fining the dendritic cell lineage. *Nat Immunol* **13**, 1145-1154 (2012).
92. S. H. Naik, P. Sathe, H. Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J. Y. Kwak, L. Wu, K. Shortman, Development of plasmacytoid and

conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* **8**, 1217-1226 (2007).
93. N. Onai, A. Obata-Onai, M. A. Schmid, T. Ohteki, D. Jarrossay, M. G. Manz, Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. *Nat Immunol* **8**, 1207-1216 (2007).
94. M. A. Schmid, D. Kingston, S. Boddupalli, M. G. Manz, Instructive cytokine signals in dendritic cell lineage commitment. *Immunol Rev* **234**, 32-44 (2010).
95. C. Waskow, K. Liu, G. Darrasse-Jeze, P. Guermonprez, F. Ginhoux, M. Merad, T. Shengelia, K. Yao, M. Nussenzweig, The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol* **9**, 676-683 (2008).
96. M. Merad, P. Sathe, J. Helft, J. Miller, A. Mortha, The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* **31**, 563-604 (2013).
97. M. Cella, D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, M. Colonna, Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* **5**, 919-923 (1999).
98. M. Gilliet, W. Cao, Y. J. Liu, Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* **8**, 594-606 (2008).
99. M. Swiecki, M. Colonna, The multifaceted biology of plasmacytoid dendritic cells. *Nat Rev Immunol* **15**, 471-485 (2015).
100. S. Sozzani, W. Vermi, A. Del Prete, F. Facchetti, Trafficking properties of plasmacytoid dendritic cells in health and disease. *Trends Immunol* **31**, 270-277 (2010).
101. J. P. Girard, C. Moussion, R. Forster, HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes. *Nat Rev Immunol* **12**, 762-773 (2012).
102. F. Facchetti, C. De Wolf-Peeters, R. De Vos, J. J. van den Oord, K. A. Pulford, V. J. Desmet, Plasmacytoid monocytes (so-called plasmacytoid T cells) in granulomatous lymphadenitis. *Hum Pathol* **20**, 588-593 (1989).
103. J. A. Villadangos, L. Young, Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* **29**, 352-361 (2008).
104. B. Reizis, A. Bunin, H. S. Ghosh, K. L. Lewis, V. Sisirak, Plasmacytoid dendritic cells: recent progress and open questions. *Annu Rev Immunol* **29**, 163-183 (2011).
105. S. Dasgupta, D. Erturk-Hasdemir, J. Ochoa-Reparaz, H. C. Reinecker, D. L. Kasper, Plasmacytoid dendritic cells mediate anti-inflammatory responses to a gut commensal molecule via both innate and adaptive mechanisms. *Cell Host Microbe* **15**, 413-423 (2014).
106. T. L. Murphy, G. E. Grajales-Reyes, X. Wu, R. Tussiwand, C. G. Briseno, A. Iwata, N. M. Kretzer, V. Durai, K. M. Murphy, Transcriptional Control of Dendritic Cell Development. *Annu Rev Immunol* **34**, 93-119 (2016).
107. S. H. Naik, D. Metcalf, A. van Nieuwenhuijze, I. Wicks, L. Wu, M. O'Keeffe, K. Shortman, Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol* **7**, 663-671 (2006).
108. J. L. Gong, K. M. McCarthy, J. Telford, T. Tamatani, M. Miyasaka, E. E. Schneeberger, Intraepithelial airway dendritic cells: a distinct subset of pulmonary dendritic cells obtained by microdissection. *J Exp Med* **175**, 797-807 (1992).
109. N. M. Beauchamp, R. Y. Busick, M. A. Alexander-Miller, Functional divergence among CD103+ dendritic cell subpopulations following pulmonary poxvirus infection. *J Virol* **84**, 10191-10199 (2010).
110. B. N. Lambrecht, H. Hammad, Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Annu Rev Immunol* **30**, 243-270 (2012).

111. A. N. Desch, G. J. Randolph, K. Murphy, E. L. Gautier, R. M. Kedl, M. H. Lahoud, I. Caminschi, K. Shortman, P. M. Henson, C. V. Jakubzick, CD103+ pulmonary dendritic cells preferentially acquire and present apoptotic cell-associated antigen. *J Exp Med* **208**, 1789-1797 (2011).
112. M. Plantinga, M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, H. Hammad, B. N. Lambrecht, Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* **38**, 322-335 (2013).
113. A. Ballesteros-Tato, B. Leon, F. E. Lund, T. D. Randall, Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. *Nat Immunol* **11**, 216-224 (2010).
114. M. L. del Rio, J. I. Rodriguez-Barbosa, E. Kremmer, R. Forster, CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells. *J Immunol* **178**, 6861-6866 (2007).
115. A. W. Ho, N. Prabhu, R. J. Betts, M. Q. Ge, X. Dai, P. E. Hutchinson, F. C. Lew, K. L. Wong, B. J. Hanson, P. A. Macary, D. M. Kemeny, Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. *J Immunol* **187**, 6011-6021 (2011).
116. M. V. Lukens, D. Kruijsen, F. E. Coenjaerts, J. L. Kimpen, G. M. van Bleek, Respiratory syncytial virus-induced activation and migration of respiratory dendritic cells and subsequent antigen presentation in the lung-draining lymph node. *J Virol* **83**, 7235-7243 (2009).
117. J. L. Coombes, K. R. Siddiqui, C. V. Arancibia-Carcamo, J. Hall, C. M. Sun, Y. Belkaid, F. Powrie, 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**, 1757-1764 (2007).
118. C. Leepiyasakulchai, L. Ignatowicz, A. Pawlowski, G. Kallenius, M. Skold, Failure to recruit anti-inflammatory CD103+ dendritic cells and a diminished CD4+ Foxp3+ regulatory T cell pool in mice that display excessive lung inflammation and increased susceptibility to Mycobacterium tuberculosis. *Infect Immun* **80**, 1128-1139 (2012).
119. K. Hildner, B. T. Edelson, W. E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B. U. Schraml, E. R. Unanue, M. S. Diamond, R. D. Schreiber, T. L. Murphy, K. M. Murphy, Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* **322**, 1097-1100 (2008).
120. D. Ashok, S. Schuster, C. Ronet, M. Rosa, V. Mack, C. Lavanchy, S. F. Marraco, N. Fasel, K. M. Murphy, F. Tacchini-Cottier, H. Acha-Orbea, Cross-presenting dendritic cells are required for control of Leishmania major infection. *Eur J Immunol* **44**, 1422-1432 (2014).
121. S. Gordon, P. R. Taylor, Monocyte and macrophage heterogeneity. *Nat Rev Immunol* **5**, 953-964 (2005).
122. M. Le Borgne, N. Etchart, A. Goubier, S. A. Lira, J. C. Sirard, N. van Rooijen, C. Caux, S. Ait-Yahia, A. Vicari, D. Kaiserlian, B. Dubois, Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity* **24**, 191-201 (2006).
123. M. H. Grayson, D. Cheung, M. M. Rohlfing, R. Kitchens, D. E. Spiegel, J. Tucker, J. T. Battaile, Y. Alevy, L. Yan, E. Agapov, E. Y. Kim, M. J. Holtzman, Induction of high-affinity IgE receptor on lung dendritic cells during viral infection leads to mucous cell metaplasia. *J Exp Med* **204**, 2759-2769 (2007).
124. H. Hammad, M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, B. N. Lambrecht, Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* **207**, 2097-2111 (2010).

125. J. L. Cruz, J. V. Perez-Giron, A. Ludtke, S. Gomez-Medina, P. Ruibal, J. Idoyaga, C. Munoz-Fontela, Monocyte-derived dendritic cells enhance protection against secondary influenza challenge by controlling the switch in CD8+ T-cell immunodominance. *Eur J Immunol* **47**, 345-352 (2017).
126. M. Samstein, H. A. Schreiber, I. M. Leiner, B. Susac, M. S. Glickman, E. G. Pamer, Essential yet limited role for CCR2(+) inflammatory monocytes during Mycobacterium tuberculosis-specific T cell priming. *Elife* **2**, e01086 (2013).
127. W. W. Reiley, M. D. Calayag, S. T. Wittmer, J. L. Huntington, J. E. Pearl, J. J. Fountain, C. A. Martino, A. D. Roberts, A. M. Cooper, G. M. Winslow, D. L. Woodland, ESAT-6-specific CD4 T cell responses to aerosol Mycobacterium tuberculosis infection are initiated in the mediastinal lymph nodes. *Proc Natl Acad Sci U S A* **105**, 10961-10966 (2008).
128. P. Andersen, J. S. Woodworth, Tuberculosis vaccines--rethinking the current paradigm. *Trends Immunol* **35**, 387-395 (2014).
129. F. Smaill, M. Jeyanathan, M. Smieja, M. F. Medina, N. Thanthrige-Don, A. Zganiacz, C. Yin, A. Heriazon, D. Damjanovic, L. Puri, J. Hamid, F. Xie, R. Foley, J. Bramson, J. Gauldie, Z. Xing, A human type 5 adenovirus-based tuberculosis vaccine induces robust T cell responses in humans despite preexisting anti-adenovirus immunity. *Sci Transl Med* **5**, 205ra134 (2013).
130. S. Afkhami, Y. Yao, Z. Xing, Methods and clinical development of adenovirus-vectored vaccines against mucosal pathogens. *Mol Ther Methods Clin Dev* **3**, 16030 (2016).
131. C. Sheridan, Gene therapy finds its niche. *Nat Biotechnol* **29**, 121-128 (2011).
132. D. Majhen, H. Calderon, N. Chandra, C. A. Fajardo, A. Rajan, R. Alemany, J. Custers, Adenovirus-based vaccines for fighting infectious diseases and cancer: progress in the field. *Human gene therapy* **25**, 301-317 (2014).
133. M. Suleman, S. Galea, F. Gavard, N. Merillon, B. Klonjkowski, E. Tartour, J. Richardson, Antigen encoded by vaccine vectors derived from human adenovirus serotype 5 is preferentially presented to CD8+ T lymphocytes by the CD8alpha+ dendritic cell subset. *Vaccine* **29**, 5892-5903 (2011).
134. J. Wang, L. Thorson, R. W. Stokes, M. Santosuosso, K. Huygen, A. Zganiacz, M. Hitt, Z. Xing, Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol* **173**, 6357-6365 (2004).
135. M. Santosuosso, S. McCormick, X. Zhang, A. Zganiacz, Z. Xing, Intranasal boosting with an adenovirus-vectored vaccine markedly enhances protection by parenteral Mycobacterium bovis BCG immunization against pulmonary tuberculosis. *Infect Immun* **74**, 4634-4643 (2006).
136. M. Santosuosso, X. Zhang, S. McCormick, J. Wang, M. Hitt, Z. Xing, Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J Immunol* **174**, 7986-7994 (2005).
137. R. Lai, S. Afkhami, S. Haddadi, M. Jeyanathan, Z. Xing, Mucosal immunity and novel tuberculosis vaccine strategies: route of immunisation-determined T-cell homing to restricted lung mucosal compartments. *European respiratory review : an official journal of the European Respiratory Society* **24**, 356-360 (2015).
138. S. Sakai, K. D. Kauffman, J. M. Schenkel, C. C. McBerry, K. D. Mayer-Barber, D. Masopust, D. L. Barber, Cutting edge: control of Mycobacterium tuberculosis infection by a subset of lung parenchyma-homing CD4 T cells. *J Immunol* **192**, 2965-2969 (2014).
139. B. J. Baaten, A. M. Cooper, S. L. Swain, L. M. Bradley, Location, Location, Location: The Impact of Migratory Heterogeneity on T Cell Function. *Front Immunol* **4**, 311 (2013).
140. N. Tatsis, H. C. Ertl, Adenoviruses as vaccine vectors. *Molecular therapy : the journal of the American Society of Gene Therapy* **10**, 616-629 (2004).

141. D. A. Muruve, The innate immune response to adenovirus vectors. *Human gene therapy* **15**, 1157-1166 (2004).
142. A. M. Cooper, S. A. Khader, The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. *Immunol Rev* **226**, 191-204 (2008).
143. M. Jeyanathan, D. Damjanovic, C. R. Shaler, R. Lai, M. Wortzman, C. Yin, A. Zganiacz, B. D. Lichty, Z. Xing, Differentially imprinted innate immunity by mucosal boost vaccination determines antituberculosis immune protective outcomes, independent of T-cell immunity. *Mucosal Immunol* **6**, 612-625 (2013).
144. V. N. Houk, J. H. Baker, K. Sorensen, D. C. Kent, The epidemiology of tuberculosis infection in a closed environment. *Arch Environ Health* **16**, 26-35 (1968).
145. A. J. Verrall, M. G. Netea, B. Alisjahbana, P. C. Hill, R. van Crevel, Early clearance of Mycobacterium tuberculosis: a new frontier in prevention. *Immunology* **141**, 506-513 (2014).
146. C. Naeslund, Experience de vaccination par le BCG dans la province de Norrbotten (Suede). *Revue de la Tuberculose* **12**, 617-636 (1931).
147. J. Kleinnijenhuis, J. Quintin, F. Preijers, L. A. Joosten, D. C. Ifrim, S. Saeed, C. Jacobs, J. van Loenhout, D. de Jong, H. G. Stunnenberg, R. J. Xavier, J. W. van der Meer, R. van Crevel, M. G. Netea, Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A* **109**, 17537-17542 (2012).
148. J. Kleinnijenhuis, J. Quintin, F. Preijers, L. A. Joosten, C. Jacobs, R. J. Xavier, J. W. van der Meer, R. van Crevel, M. G. Netea, BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol* **155**, 213-219 (2014).
149. K. Yoshida, T. Maekawa, Y. Zhu, C. Renard-Guillet, B. Chatton, K. Inoue, T. Uchiyama, K. Ishibashi, T. Yamada, N. Ohno, K. Shirahige, M. Okada-Hatakeyama, S. Ishii, The transcription factor ATF7 mediates lipopolysaccharide-induced epigenetic changes in macrophages involved in innate immunological memory. *Nat Immunol* **16**, 1034-1043 (2015).
150. P. Andersen, S. H. Kaufmann, Novel vaccination strategies against tuberculosis. *Cold Spring Harb Perspect Med* **4**, (2014).
151. A. K. Tyagi, P. Nangpal, V. Satchidanandam, Development of vaccines against tuberculosis. *Tuberculosis (Edinb)* **91**, 469-478 (2011).
152. R. Madan-Lala, J. K. Sia, R. King, T. Adekambi, L. Monin, S. A. Khader, B. Pulendran, J. Rengarajan, Mycobacterium tuberculosis impairs dendritic cell functions through the serine hydrolase Hip1. *J Immunol* **192**, 4263-4272 (2014).
153. M. Divangahi, S. M. Behar, H. Remold, Dying to live: how the death modality of the infected macrophage affects immunity to tuberculosis. *Adv Exp Med Biol* **783**, 103-120 (2013).
154. A. Bohsali, H. Abdalla, K. Velmurugan, V. Briken, The non-pathogenic mycobacteria M. smegmatis and M. fortuitum induce rapid host cell apoptosis via a caspase-3 and TNF dependent pathway. *BMC Microbiol* **10**, 237 (2010).
155. F. A. Post, C. Manca, O. Neyrolles, B. Ryffel, D. B. Young, G. Kaplan, Mycobacterium tuberculosis 19-kilodalton lipoprotein inhibits Mycobacterium smegmatis-induced cytokine production by human macrophages in vitro. *Infect Immun* **69**, 1433-1439 (2001).
156. N. Majumder, S. Bhattacharjee, R. Dey, S. Bhattacharyya Majumdar, N. K. Pal, S. Majumdar, Arabinosylated lipoarabinomannan modulates the impaired cell mediated immune response in Mycobacterium tuberculosis H37Rv infected C57BL/6 mice. *Microbes Infect* **10**, 349-357 (2008).
157. D. Chatterjee, K. Lowell, B. Rivoire, M. R. McNeil, P. J. Brennan, Lipoarabinomannan of Mycobacterium tuberculosis. Capping with mannosyl residues in some strains. *J Biol Chem* **267**, 6234-6239 (1992).

158. J. M. Richmond, E. R. Duffy, J. Lee, K. Kaboli, Y. S. Kim, D. G. Remick, H. Kornfeld, W. W. Cruikshank, Mannose-capped Lipoarabinomannan from Mycobacterium tuberculosis induces soluble tumor necrosis factor receptor production through tumor necrosis factor alpha-converting enzyme activation. *Infect Immun* **80**, 3858-3868 (2012).
159. B. S. Thompson, P. M. Chilton, J. R. Ward, J. T. Evans, T. C. Mitchell, The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. *J Leukoc Biol* **78**, 1273-1280 (2005).
160. C. N. Baxevanis, I. F. Voutsas, O. E. Tsitsilonis, Toll-like receptor agonists: current status and future perspective on their utility as adjuvants in improving anticancer vaccination strategies. *Immunotherapy* **5**, 497-511 (2013).
161. A. A. Ashkar, K. L. Mossman, B. K. Coombes, C. L. Gyles, R. Mackenzie, FimH adhesin of type 1 fimbriae is a potent inducer of innate antimicrobial responses which requires TLR4 and type 1 interferon signalling. *PLoS Pathog* **4**, e1000233 (2008).
162. M. F. Abdul-Careem, M. Firoz Mian, A. E. Gillgrass, M. J. Chenoweth, N. G. Barra, T. Chan, A. A. Al-Garawi, M. V. Chew, G. Yue, N. van Roojen, Z. Xing, A. A. Ashkar, FimH, a TLR4 ligand, induces innate antiviral responses in the lung leading to protection against lethal influenza infection in mice. *Antiviral Res* **92**, 346-355 (2011).
163. J. W. Williams, M. Y. Tjota, B. S. Clay, B. Vander Lugt, H. S. Bandukwala, C. L. Hrusch, D. C. Decker, K. M. Blaine, B. R. Fixsen, H. Singh, R. Sciammas, A. I. Sperling, Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nature communications* **4**, 2990 (2013).
164. A. Schlitzer, N. McGovern, P. Teo, T. Zelante, K. Atarashi, D. Low, A. W. Ho, P. See, A. Shin, P. S. Wasan, G. Hoeffel, B. Malleret, A. Heiseke, S. Chew, L. Jardine, H. A. Purvis, C. M. Hilkens, J. Tam, M. Poidinger, E. R. Stanley, A. B. Krug, L. Renia, B. Sivasankar, L. G. Ng, M. Collin, P. Ricciardi-Castagnoli, K. Honda, M. Haniffa, F. Ginhoux, IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* **38**, 970-983 (2013).
165. M. Mashayekhi, M. M. Sandau, I. R. Dunay, E. M. Frickel, A. Khan, R. S. Goldszmid, A. Sher, H. L. Ploegh, T. L. Murphy, L. D. Sibley, K. M. Murphy, CD8alpha(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. *Immunity* **35**, 249-259 (2011).
166. B. D. Medoff, E. Seung, S. Hong, S. Y. Thomas, B. P. Sandall, J. S. Duffield, D. A. Kuperman, D. J. Erle, A. D. Luster, CD11b+ myeloid cells are the key mediators of Th2 cell homing into the airway in allergic inflammation. *J Immunol* **182**, 623-635 (2009).
167. Y. Gao, S. A. Nish, R. Jiang, L. Hou, P. Licona-Limon, J. S. Weinstein, H. Zhao, R. Medzhitov, Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* **39**, 722-732 (2013).
168. A. T. Satpathy, C. G. Briseno, J. S. Lee, D. Ng, N. A. Manieri, W. Kc, X. Wu, S. R. Thomas, W. L. Lee, M. Turkoz, K. G. McDonald, M. M. Meredith, C. Song, C. J. Guidos, R. D. Newberry, W. Ouyang, T. L. Murphy, T. S. Stappenbeck, J. L. Gommerman, M. C. Nussenzweig, M. Colonna, R. Kopan, K. M. Murphy, Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat Immunol* **14**, 937-948 (2013).
169. M. Martinez-Lopez, S. Iborra, R. Conde-Garrosa, D. Sancho, Batf3-dependent CD103+ dendritic cells are major producers of IL-12 that drive local Th1 immunity against Leishmania major infection in mice. *Eur J Immunol* **45**, 119-129 (2015).
170. S. Bajana, S. Turner, J. Paul, E. Ainsua-Enrich, S. Kovats, IRF4 and IRF8 Act in CD11c+ Cells To Regulate Terminal Differentiation of Lung Tissue Dendritic Cells. *J Immunol* **196**, 1666-1677 (2016).

171. B. Vander Lugt, A. A. Khan, J. A. Hackney, S. Agrawal, J. Lesch, M. Zhou, W. P. Lee, S. Park, M. Xu, J. DeVoss, C. J. Spooner, C. Chalouni, L. Delamarre, I. Mellman, H. Singh, Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat Immunol* **15**, 161-167 (2014).
172. O. Annacker, J. L. Coombes, V. Malmstrom, H. H. Uhlig, T. Bourne, B. Johansson-Lindbom, W. W. Agace, C. M. Parker, F. Powrie, Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* **202**, 1051-1061 (2005).
173. K. A. Bodnar, N. V. Serbina, J. L. Flynn, Fate of Mycobacterium tuberculosis within murine dendritic cells. *Infect Immun* **69**, 800-809 (2001).
174. L. Tailleux, O. Neyrolles, S. Honore-Bouakline, E. Perret, F. Sanchez, J. P. Abastado, P. H. Lagrange, J. C. Gluckman, M. Rosenzwajg, J. L. Herrmann, Constrained intracellular survival of Mycobacterium tuberculosis in human dendritic cells. *J Immunol* **170**, 1939-1948 (2003).
175. X. Hao, T. S. Kim, T. J. Braciale, Differential response of respiratory dendritic cell subsets to influenza virus infection. *J Virol* **82**, 4908-4919 (2008).
176. M. Guilliams, B. N. Lambrecht, H. Hammad, Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections. *Mucosal Immunol* **6**, 464-473 (2013).
177. S. Redpath, P. Ghazal, N. R. Gascoigne, Hijacking and exploitation of IL-10 by intracellular pathogens. *Trends Microbiol* **9**, 86-92 (2001).
178. B. Everts, R. Tussiwand, L. Dreesen, K. C. Fairfax, S. C. Huang, A. M. Smith, C. M. O'Neill, W. Y. Lam, B. T. Edelson, J. F. Urban, Jr., K. M. Murphy, E. J. Pearce, Migratory CD103+ dendritic cells suppress helminth-driven type 2 immunity through constitutive expression of IL-12. *J Exp Med* **213**, 35-51 (2016).
179. I. Satti, J. Meyer, S. A. Harris, Z. R. Manjaly Thomas, K. Griffiths, R. D. Antrobus, R. Rowland, R. L. Ramon, M. Smith, S. Sheehan, H. Bettinson, H. McShane, Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. *The Lancet. Infectious diseases* **14**, 939-946 (2014).
180. M. Jeyanathan, Z. Shao, X. Yu, R. Harkness, R. Jiang, J. Li, Z. Xing, T. Zhu, AdHu5Ag85A Respiratory Mucosal Boost Immunization Enhances Protection against Pulmonary Tuberculosis in BCG-Primed Non-Human Primates. *PLoS One* **10**, e0135009 (2015).
181. Z. Xing, C. T. McFarland, J. M. Sallenave, A. Izzo, J. Wang, D. N. McMurray, Intranasal mucosal boosting with an adenovirus-vectored vaccine markedly enhances the protection of BCG-primed guinea pigs against pulmonary tuberculosis. *PLoS One* **4**, e5856 (2009).
182. G. S. Dean, D. Clifford, A. O. Whelan, E. Z. Tchilian, P. C. Beverley, F. J. Salguero, Z. Xing, H. M. Vordermeier, B. Villarreal-Ramos, Protection Induced by Simultaneous Subcutaneous and Endobronchial Vaccination with BCG/BCG and BCG/Adenovirus Expressing Antigen 85A against Mycobacterium bovis in Cattle. *PLoS One* **10**, e0142270 (2015).
183. Z. Mikhak, J. P. Strassner, A. D. Luster, Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. *J Exp Med* **210**, 1855-1869 (2013).
184. S. J. Ray, S. N. Franki, R. H. Pierce, S. Dimitrova, V. Koteliansky, A. G. Sprague, P. C. Doherty, A. R. de Fougerolles, D. J. Topham, The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* **20**, 167-179 (2004).
185. A. R. Ferguson, V. H. Engelhard, CD8 T cells activated in distinct lymphoid organs differentially express adhesion proteins and coexpress multiple chemokine receptors. *J Immunol* **184**, 4079-4086 (2010).

186. M. G. Netea, L. A. Joosten, E. Latz, K. H. Mills, G. Natoli, H. G. Stunnenberg, L. A. O'Neill, R. J. Xavier, Trained immunity: A program of innate immune memory in health and disease. *Science* **352**, aaf1098 (2016).

# Appendix. Mucosal immunity and novel tuberculosis vaccine strategies: route of immunisation determined T-cell homing to restricted lung mucosal compartments

**Title of Review**: Mucosal immunity and novel tuberculosis vaccine strategies: route of immunisation determined T-cell homing to restricted lung mucosal compartments

**Rocky Lai**, Sam Afkhami, Siamak Haddadi, Manglakumairi Jeyanathan, Zhou Xing

Published in *European Respiratory Review* 2015. 24(136): 356-60.

# Mucosal immunity and novel tuberculosis vaccine strategies: route of immunisation-determined T-cell homing to restricted lung mucosal compartments

Rocky Lai, Sam Afkhami, Siamak Haddadi, Mangalakumari Jeyanathan and Zhou Xing

**Affiliation**: McMaster Immunology Research Centre, M. G. DeGroote Institute for Infectious Disease Research, and Dept of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada.

**Correspondence**: Zhou Xing, Dept of Pathology and Molecular Medicine, and McMaster Immunology Research Centre, McMaster University, 1280 Main Street West, Room MDCL-4012, Hamilton, ON, L8S 4K1, Canada. E-mail: xingz@mcmaster.ca

ABSTRACT Despite the use of bacille Calmette–Guérin (BCG) for almost a century, pulmonary tuberculosis (TB) continues to be a serious global health concern. Therefore, there has been a pressing need for the development of new booster vaccines to enhance existing BCG-induced immunity. Protection following mucosal intranasal immunisation with AdHu5Ag85A is associated with the localisation of antigen-specific T-cells to the lung airway. However, parenteral intramuscular immunisation is unable to provide protection despite the apparent presence of antigen-specific T-cells in the lung interstitium. Recent advances in intravascular staining have allowed us to reassess the previously established T-cell distribution profile and its relationship with the observed differential protection. Respiratory mucosal immunisation empowers T-cells to home to both the lung interstitium and the airway lumen, whereas intramuscular immunisation-activated T-cells are largely trapped within the pulmonary vasculature, unable to populate the lung interstitium and airway. Given the mounting evidence supporting the safety and enhanced efficacy of respiratory mucosal immunisation over the traditional parenteral immunisation route, a greater effort should be made to clinically develop respiratory mucosal-deliverable TB vaccines.

@ERSpublications
**Immunisation route determines TB vaccine efficacy based on whether T-cells can enter restricted lung mucosal sites** http://ow.ly/M0shT

## Introduction

*Mycobacterium tuberculosis* is the causative agent of pulmonary tuberculosis (TB) and remains the leading infectious cause of death [1]. Although control of primary *M. tuberculosis* infection in the lung is strongly correlated with the induction of T-helper cell 1 cellular responses within the lungs, such responses are delayed for up to 3 weeks post-infection [2, 3]. This leads to unchecked bacterial infection within the host [4]. The only clinically approved TB vaccine, bacille Calmette–Guérin (BCG), accelerates the emergence of T-cell responses in the lung by only a few days [3, 5]. Furthermore, BCG-induced immunity wanes over time, so that by adulthood most people are no longer protected from TB. Therefore, the development of vaccines able to further accelerate and enhance existing BCG-mediated T-cell responses has been recognised as a priority in the field of TB vaccine development [6]. To date, there are over a dozen candidate TB vaccines at various stages of testing in the clinical trial pipeline [6], either designed to replace BCG itself or

Received: March 24 2015 | Accepted after revision: April 21 2015

Conflict of interest: None declared.

Provenance: Submitted article, peer reviewed.

to boost pre-existing BCG responses. In particular, viral-based booster vaccines are among those that have demonstrated great promise in clinical trials [7–9].

## Protection against pulmonary TB is determined by the differential distribution of T-cells in lung anatomic compartments following immunisation

Since the lung is the site of *M. tuberculosis* entry, examination of immune responses in the lung is of particular importance. Upon exposure to *M. tuberculosis*, the bacterium is deposited into the lung airway, and infects local macrophage and dendritic cells. After 8–9 days these cells migrate to the lung draining lymph node, where they prime naïve T-cells and provide the activation signals to generate a population of *M. tuberculosis*-specific T-cells [10]. These cells then travel through the lymphatic circulation into the venous blood circulation, eventually ending up in the pulmonary vasculature (pulmonary artery). Activated antigen-specific T-cells then migrate through the endothelium into the pulmonary interstitium (parenchyma), eventually entering the lung airways where they become long-lived effector memory cells that are able to activate infected macrophage populations for enhanced control of *M. tuberculosis* infection [2, 3].

The current knowledge in the field is that the presence of anti-TB T-cells induced by immunisation in the lung airways before or shortly after *M. tuberculosis* infection is critical for protection [5, 11]. In this regard, the respiratory mucosal route of immunisation is superior to the parenteral route in protecting against pulmonary TB [12, 13], because of its ability to induce anti-TB T-cell immunity in the lung airways in addition to inducing T-cell responses in the lung interstitium. In contrast, parenteral immunisation fails to elicit T-cell responses in the airway lumen although it induces T-cells to populate the lung interstitium [12].

Paradoxically, our previous work has demonstrated that despite the generation of fully functional antigen-specific T-cells in the peripheral lymphoid tissues and the lung interstitium, intramuscular immunisation fails to protect against pulmonary TB [14]. It was initially believed that the inability of intramuscular immunisation to protect against pulmonary TB was linked to the absence of T-cells in the airway, which are present following intranasal immunisation. Indeed, adoptive transfer of CD8 T-cells from the spleen of intramuscularly immunised animals into the airway of naïve SCID (severe combined immunodeficiency) mice confers protection against pulmonary TB, highlighting the critical importance of T-cell localisation within the airway for anti-TB immunity [13]. These data, in conjunction with the initial observation that both intramuscular and intranasal immunisation were able to induce anti-TB immunity in the lung as a whole [12], challenge the stereotypical view that the presence of TB-reactive T-cells in the lung before infection equates to protection against pulmonary TB. In other words, the presence of antigen-specific T-cells in the lung interstitium following intramuscular immunisation does not explain the complete lack of protection in the animals immunised *via* intramuscular route.

The view that the presence of TB-reactive T-cells in the lung is critical for protection ascends from consideration of the lung as a single tissue compartment. In recent years, discrimination of the lung interstitium from the lung airways, by us and others, has led to better understanding of the anatomic distribution of T-cell responses in the lung and its relevance to anti-TB protection. In addition to these two major lung mucosal compartments, however, there is a dense network of pulmonary capillaries underlying the alveoli that forms a third major compartment in the lung [15]. The lung is one of the most highly vascularised organs and holds ~40% of the total body blood volume at any given time. Thus, blood-borne leukocyte contamination of the lung tissue may have hindered the accurate interpretation of T-cell responses in the lung as a whole or in the lung interstitium.

Historically, a common approach to get around to this problem in animal models is to perfuse the lung *via* the pulmonary artery before lung mononuclear cell isolation. Our group and others have employed this approach to "exclude" blood-borne leukocytes [5, 12, 13]. However, an intravascular staining approach was recently used to demonstrate that perfusion was far from effective in removing T-cells from the pulmonary vasculature. In fact, up to 97% of the CD8 T-cells thought to be located in the lung interstitium were present in the lung vasculature following a respiratory viral infection [16]. This observation questions the previous conclusions, including our own, and necessitates revisiting the anatomic distribution of lung T-cell responses following different routes of immunisation.

The availability of the recently developed technique of intravascular staining helps to discriminate pulmonary intravascular and lung tissue T-cell populations for the first time [16, 17], and appreciate their relationship with anti-TB protection. In our previous studies we have reported that, regardless of route of immunisation, a large population of anti-TB T-cells can be isolated from perfused mouse lungs [12, 13]. However, when the tissue compartment is discriminated from the vasculature we have found that it is only following intranasal immunisation that antigen-specific T-cells are located primarily within the lung interstitium and airways. In stark contrast, following intramuscular immunisation the majority of such cells are confined to the pulmonary vasculature (unpublished data) and are not present within the lung

DOI: 10.1183/16000617.00002515

interstitium (fig. 1). The anatomic distribution of T-cells following parenteral immunisation resembles the T-cell distribution in the lung of naïve mice, where 99% of lymphocytes are confined to pulmonary vasculature. These preliminary findings, therefore, provide an updated understanding of the basic mechanisms regarding the localisation of T-cells following different routes of immunisation. Importantly, these findings challenge the conventional concept that the lung interstitium alongside the peripheral lymphoid tissues is a common or unrestricted site for antigen-experienced T-cells to home to.

Interestingly, profiling a variety of chemokine receptors and integrins for their mRNA expression on antigen-experienced T-cells isolated from the total lung of mice immunised *via* either the respiratory or parenteral route with a viral-vectored TB vaccine also displayed striking differences. Expression of chemokine receptors CCR1, CCR6 and CCR8, and integrins CD103 ($\alpha_e\beta_7$) and VLA-1 ($\alpha_1\beta_1$) were many fold increased on antigen-specific T-cells induced by respiratory mucosal immunisation compared with those induced by intramuscular vaccination (unpublished data) (fig. 1). Furthermore, flow cytometry analysis of proteins for these genes on antigen-specific T-cells residing in the lung interstitium and airway lumen following respiratory mucosal immunisation revealed similar expression patterns for these markers on these two populations of T-cells, further supporting that T-cell entry to the lung interstitium is as restricted as entry to the airway lumen. CD103 and VLA-1 expression has been defined as a residential surface marker for the T-cells in nonlymphoid tissues [18–20]. Currently, homing molecules involved in migration of antigen-experienced T-cells to the lung are not well established [21]. This is partly due to the lack of techniques to discriminate intravascular and tissue residing T-cells in the past. Use of the intravascular staining approach will facilitate redefinition of the homing molecules involved in T-cell recruitment to and retention within the lung interstitium and airway lumen.

## Effect of local pro-inflammatory signals on the distribution of T-cells in lung anatomic compartments

The factors that drive the entry of T-cells into different lung mucosal compartments following respiratory mucosal immunisation remain unclear. However, it is well established that innate immune responses play

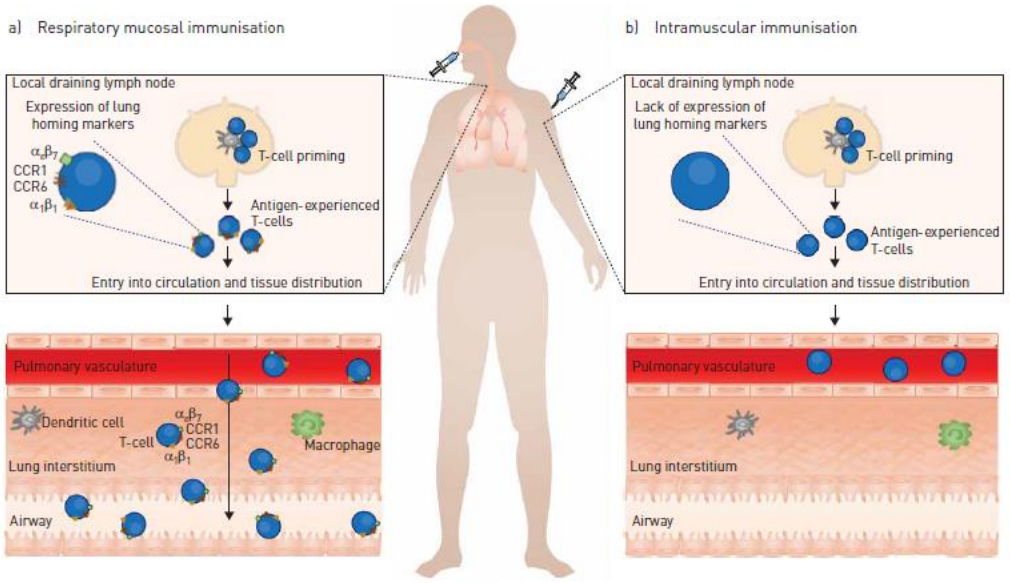



FIGURE 1 Route of immunisation determines whether anti-tuberculosis (TB) T-cells acquire the ability to home to the restricted lung mucosal compartments. Protection against pulmonary TB is associated with the ability of anti-TB T-cells to exit the pulmonary vasculature and gain entry into the restricted lung mucosal compartments. a) T-cells primed within the lung local draining lymph nodes following respiratory mucosal immunisation express various lung homing molecules including $\alpha_e\beta_7$ (CD103), CCR1, CCR6 and $\alpha_1\beta_1$ (VLA-1). These antigen-experienced T-cells, primed with various lung homing markers, are able to exit the pulmonary vasculature and gain entry into the lung interstitium and airway lumen. Once localised at the site of infection, these cells are able to provide rapid protection against pulmonary TB. b) T-cells primed following parenteral immunisation lack expression of lung homing molecules. Following priming in the local draining lymph nodes, these cells are restricted to the pulmonary vasculature, thus limiting their ability to provide robust anti-TB immunity.


DOI: 10.1183/16000617.00002515

a large role in the instruction and induction of adaptive T-cell responses. Following immunisation, local dendritic cell populations acquire antigens and subsequently migrate to the local draining lymph node, priming and activating naïve T-cells. It has been suggested that such antigen-presenting cells play an important role in influencing the expression of receptors critical to the migration of T-cells (termed migratory receptors) to specific tissue sites (reviewed by Baaten *et al.* [21]).

The anatomical location from which these dendritic cells are derived also seems to influence the trafficking of activated T-cells to specific tissue sites. Extensive evidence demonstrates the ability of local dendritic cell populations to influence the migration of T-cells to the gut and skin [22–25]. Whether this also holds true for T-cell migration to the respiratory tract is less clear. However, a recent study demonstrated that lung dendritic cells were able to preferentially recruit T-cells back to the lung in comparison with dendritic cells derived from other local sites following exposure to inhaled antigens through the induction of CCR4 [26]. However, it is likely that there are other surface molecules involved. In this regard CD8 T-cells primed in the lung draining lymph node were enriched for CCR5 and CXCR3 expression [27]. Furthermore, others have demonstrated that following intranasal vaccination with AdHu5Ag85A, antigen-specific CD8 T-cells localised to the lung expressed high levels of CXCR6 [28]. In addition, we have found CCR1, CCR6, CCR8, CD103 and VLA-1 to be upregulated on activated T-cells by respiratory mucosal, but not by intramuscular, immunisation (unpublished data), suggesting the involvement of multiple molecules in T-cell homing into the lung interstitium and airway. It is tempting to speculate that respiratory mucosal immunisation preferentially activates certain subsets of lung dendritic cells that are able to instruct T-cells to home to the lung interstitium and airway where they are needed.

The superior T-cell responses induced within the lung interstitium and airway lumen by intranasal immunisation may also be attributed to the ability of the viral vaccine backbone to activate the soluble innate immune signals in the local lung microenvironment. Adenoviruses are highly immunogenic, and respiratory mucosal immunisation with AdHu5Ag85A may activate a wide variety of lung local immune responses [29, 30]. In particular, the AdHu5 vector has been shown to elicit potent pro-inflammatory responses including the release of tumour necrosis factor-α, interleukin (IL)-6 and IL-12, as well as the recruitment and activation of macrophages and dendritic cells to the site of infection (unpublished data and [29, 31]). Indeed, the immunogenic nature of AdHu5 vectors is what makes them an attractive vaccine delivery platform. However, while both intranasal and intramuscular administrations of the vaccine are able to activate local innate responses, only intranasal mucosal immunisation effectively activates local lung innate immune responses. Indeed, we have previously demonstrated that the inoculation of pro-inflammatory agonists, such as Toll-like receptor ligands, into the lung of intramuscularly immunised animals is able to draw antigen-specific T-cells from the peripheral sites into the airway lumen and provide protection against pulmonary TB [5, 14]. Furthermore, we have recently demonstrated that a robust innate inflammatory response is key for the timely generation of anti-TB T-cell immunity in the lung following primary pulmonary *M. tuberculosis* infection [10].

## Respiratory mucosal vaccination in human clinical trials

There are ongoing efforts for evaluation of respiratory mucosal vaccination against other respiratory pathogens. In particular, pulmonary delivery of an intranasal live attenuated influenza vaccine has demonstrated high efficacy in children in comparison with the injectable trivalent vaccine [32]. In addition, there have been attempts to explore pulmonary delivery of measles vaccine as an alternative to boost immunity, and this has been demonstrated to be safe and more immunogenic than the injected measles vaccine [33, 34].

With regards to TB vaccines, most current candidates have been developed for and evaluated *via* the parenteral route of immunisation. MVAAg85A represents one of the most advanced TB vaccine candidates to date. Although the recent phase IIb efficacy trial in South Africa demonstrated that the parenteral route of boosting was ineffective in enhancing protection in BCG-primed infants [9], the demonstrated safety and immunogenicity in a recent phase I MVAAg85A aerosol trial has provided the rationale, and optimism, for respiratory mucosal delivery of a TB vaccine in humans [35].

## Conclusion

Our understanding of what may constitute an effective TB vaccination strategy has increased significantly since BCG was first introduced into human immunisation programmes. The effective boost TB vaccination strategies should aim to generate is memory T-cells that are able to home to the two restricted lung mucosal compartments: the respiratory mucosal surface and the lung interstitium (parenchyma). Such local mucosal tissue-associated T-cells and systemically located T-cells generated by parenteral BCG priming will together provide both local and systemic protection against pulmonary TB. The respiratory mucosal route of immunisation represents the most effective way to generate T-cells capable of homing to the restricted lung mucosal compartments. In comparison, the parenteral route of immunisation activates

DOI: 10.1183/16000617.00002515

T-cells that are largely trapped only within the pulmonary vasculature and are thus unable to migrate into the site of action, particularly in the early stage of *M. tuberculosis* infection. Increasing numbers of clinical studies to evaluate the respiratory mucosal delivery of TB vaccines are expected in the next few years.

## References


1 World Health Organization. Global Tuberculosis Report 2014. Geneva, WHO Press, 2014.
2 Shaler CR, Horvath C, Lai R, *et al.* Understanding delayed T-cell priming, lung recruitment, and airway luminal T-cell responses in host defense against pulmonary tuberculosis. *Clin Dev Immunol* 2012; 2012: 628293.
3 Cooper AM. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol* 2009; 27: 393–422.
4 Davenport MP, Belz GT, Ribeiro RM. The race between infection and immunity: how do pathogens set the pace? *Trends Immunol* 2009; 30: 61–66.
5 Horvath CN, Shaler CR, Jeyanathan M, *et al.* Mechanisms of delayed anti-tuberculosis protection in the lung of parenteral BCG-vaccinated hosts: a critical role of airway luminal T cells. *Mucosal Immunol* 2012; 5: 420–431.
6 Xing Z, Jeyanathan M, Smaill F. New approaches to TB vaccination. *Chest* 2014; 146: 804–812.
7 Xing Z, Lichty BD. Use of recombinant virus-vectored tuberculosis vaccines for respiratory mucosal immunization. *Tuberculosis (Edinb)* 2006; 86: 211–217.
8 Smaill F, Jeyanathan M, Smieja M, *et al.* A human type 5 adenovirus-based tuberculosis vaccine induces robust T cell responses in humans despite preexisting anti-adenovirus immunity. *Sci Transl Med* 2013; 5: 205ra134.
9 Tameris MD, Hatherill M, Landry BS, *et al.* Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. *Lancet* 2013; 381: 1021–1028.
10 Lai R, Jeyanathan M, Shaler CR, *et al.* Restoration of innate immune activation accelerates Th1-cell priming and protection following pulmonary mycobacterial infection. *Eur J Immunol* 2014; 44: 1375–1386.
11 Jeyanathan M, Heriazon A, Xing Z. Airway luminal T cells: a newcomer on the stage of TB vaccination strategies. *Trends Immunol* 2010; 31: 247–252.
12 Wang J, Thorson L, Stokes RW, *et al.* Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol* 2004; 173: 6357–6365.
13 Santosuosso M, Zhang X, McCormick S, *et al.* Mechanisms of mucosal and parenteral tuberculosis vaccinations: adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J Immunol* 2005; 174: 7986–7994.
14 Santosuosso M, McCormick S, Roediger E, *et al.* Mucosal luminal manipulation of T cell geography switches on protective efficacy by otherwise ineffective parenteral genetic immunization. *J Immunol* 2007; 178: 2387–2395.
15 Tschernig T, Pabst R. What is the clinical relevance of different lung compartments? *BMC Pulm Med* 2009; 9: 39.
16 Anderson KG, Sung H, Skon CN, *et al.* Cutting edge: intravascular staining redefines lung CD8 T cell responses. *J Immunol* 2012; 189: 2702–2706.
17 Sakai S, Kauffman KD, Schenkel JM, *et al.* Cutting edge: control of *Mycobacterium tuberculosis* infection by a subset of lung parenchyma-homing CD4 T cells. *J Immunol* 2014; 192: 2965–2969.
18 Mackay LK, Rahimpour A, Ma JZ, *et al.* The developmental pathway for $CD103^{+}CD8^{+}$ tissue-resident memory T cells of skin. *Nat Immunol* 2013; 14: 1294–1301.
19 Annacker O, Coombes JL, Malmstrom V, *et al.* Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* 2005; 202: 1051–1061.
20 Wakim LM, Gupta N, Mintern JD, *et al.* Enhanced survival of lung tissue-resident memory $CD8^{+}$ T cells during infection with influenza virus due to selective expression of IFITM3. *Nat Immunol* 2013; 14: 238–245.
21 Baaten BJ, Cooper AM, Swain SL, *et al.* Location, location, location: the impact of migratory heterogeneity on T cell function. *Front Immunol* 2013; 4: 311.
22 Iwata M, Hirakiyama A, Eshima Y, *et al.* Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 2004; 21: 527–538.
23 McCully ML, Ladell K, Hakobyan S, *et al.* Epidermis instructs skin homing receptor expression in human T cells. *Blood* 2012; 120: 4591–4598.
24 McCully ML, Moser B. The human cutaneous chemokine system. *Front Immunol* 2011; 2: 33.
25 Mora JR, Bono MR, Manjunath N, *et al.* Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature* 2003; 424: 88–93.
26 Mikhak Z, Strassner JP, Luster AD. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. *J Exp Med* 2013; 210: 1855–1869.
27 Ferguson AR, Engelhard VH. CD8 T cells activated in distinct lymphoid organs differentially express adhesion proteins and coexpress multiple chemokine receptors. *J Immunol* 2010; 184: 4079–4086.
28 Lee LN, Ronan EO, de Lara C, *et al.* CXCR6 is a marker for protective antigen-specific cells in the lungs after intranasal immunization against *Mycobacterium tuberculosis*. *Infect Immun* 2011; 79: 3328–3337.
29 Tatsis N, Ertl HC. Adenoviruses as vaccine vectors. *Mol Ther* 2004; 10: 616–629.
30 Muruve DA. The innate immune response to adenovirus vectors. *Hum Gene Ther* 2004; 15: 1157–1166.
31 Jeyanathan M, Damjanovic D, Shaler CR, *et al.* Differentially imprinted innate immunity by mucosal boost vaccination determines antituberculosis immune protective outcomes, independent of T-cell immunity. *Mucosal Immunol* 2013; 6: 612–625.
32 Ambrose CS, Wu X, Knuf M, *et al.* The efficacy of intranasal live attenuated influenza vaccine in children 2 through 17 years of age: a meta-analysis of 8 randomized controlled studies. *Vaccine* 2012; 30: 886–892.
33 Dilraj A, Cutts FT, de Castro JF, *et al.* Response to different measles vaccine strains given by aerosol and subcutaneous routes to schoolchildren: a randomised trial. *Lancet* 2000; 355: 798–803.
34 Dilraj A, Sukhoo R, Cutts FT, *et al.* Aerosol and subcutaneous measles vaccine: measles antibody responses 6 years after re-vaccination. *Vaccine* 2007; 25: 4170–4174.
35 Satti I, Meyer J, Harris SA, *et al.* Safety and immunogenicity of a candidate tuberculosis vaccine MVA85A delivered by aerosol in BCG-vaccinated healthy adults: a phase 1, double-blind, randomised controlled trial. *Lancet Infect Dis* 2014; 14: 939–946.



DOI: 10.1183/16000617.00002515