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UMinho | 2022

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Tese de Doutoramento
Doutoramento em Envelhecimento e Doenças Crónicas

Trabalho efetuado sob a orientação do
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e da
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abril de 2022

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

ACKNOWLEDGMENTS

Em primeiro lugar, quero agradecer ao meu orientador Egídio Torrado. Obrigada por todos os ensinamentos, apoio e confiança que me transmitiste durante estes anos. À minha orientadora, Helena Soares, obrigada pelo apoio e motivação. Ao professor Gil Castro, agradeço todos os conselhos, boa-disposição e confiança depositada em mim. Foi, sem dúvida, uma mais-valia neste percurso. A todos os membros, representantes e unidades orgânicas do ICVS/EM pelo apoio prestado, obrigada.

Quero agradecer de forma muito especial à Margarida e à Carolina. Vocês foram o meu refúgio, a minha motivação e as minhas confidentes durante estes anos. Sem vocês teria sido muito mais difícil. Merecem tudo e irão comigo para a vida. Quero agradecer aos meus colegas da última edição do PhDOC pela prontidão em ajudar. Em especial, ao João do Canto que me acompanha desde os tempos de mestrado e com quem consigo sempre uma gargalhada bem dada. À minha parceira Alex por ser tão prestável e motivadora. Espero conseguir crescer com um espírito tão jovem quanto o teu! À Cláudia e à Palmira, obrigada pelos conselhos de vida e de ciência que sempre me deram. São, sem dúvida, um exemplo para mim. Quero também agradecer à Belém por ser uma força da natureza e estar sempre motivada em tudo o que faz, isso tem um impacto muito positivo nas pessoas que te rodeiam. Aos amigos que me acompanharam neste percurso. Obrigada!

Aos meus pais, tios, primos e avós, obrigada por verem sempre o melhor de mim e por terem essa confiança inabalável nas minhas decisões, mesmo quando eu própria não a tenho. Ao Diogo, que já quase tudo viveu comigo, obrigada pela paciência, pelo apoio e pelo carinho. Sou mesmo uma sortuda.

FINANCIAL SUPPORT

The work presented in this thesis was developed in the Life and Health Sciences Research Institute (ICVS), University of Minho. Financial support was provided by grants from the Foundation for Science and Technology (FCT) PD/BD/137447/2018, PTDC/MED-ONC/28658/2017, PTDC/SAU-INF/28463/2017, UIDB/50026/2020, and UIDP/50026/2020; ICVS Scientific Microscopy Platform, member of the national infrastructure PPBI - Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122); and the projects NORTE-01-0145-FEDER-000013, NORTE-01-0145-FEDER-000023, and supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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RESUMO

Regulação da resposta pulmonar das células T CD4+ durante a infeção com *Mycobacterium tuberculosis*

A pandemia de COVID-19 reverteu os sucessos de anos anteriores no que diz respeito ao fornecimento de serviços essenciais de combate à tuberculose e ao decréscimo da sua incidência. Pela primeira vez em mais de uma década, a mortalidade da tuberculose aumentou, e estima-se que atingirá as 1,4 milhões de mortes até 2025. Portanto, são urgentes avanços científicos que contribuam para mitigar estes efeitos. O controlo da infeção por *Mycobacterium tuberculosis* (Mtb) requer a indução de uma resposta inflamatória das células T sem que isto resulte em danos para o funcionamento dos tecidos. Portanto, perceber os mecanismos que governam o equilíbrio entre proteção e imunopatologia é extremamente relevante. A interleucina (IL)-10 é um importante regulador imunológico e a sua expressão em pulmões infetados com Mtb tem sido descrita como cooperante na esterilização dos granulomas. Contudo, a IL-10 está também associada a suscetibilidade, ainda que os mecanismos através dos quais ela desempenha as suas funções no contexto da tuberculose não sejam conhecidos. Nesta tese de doutoramento, eu descrevo o impacto que níveis elevados de IL-10, após uma infeção com Mtb, tem na resposta pulmonar de células T CD4+. Níveis elevados de IL-10 durante uma infeção crónica não interferem no controlo da infeção. Contudo, níveis elevados de IL-10 num período anterior ao desenvolvimento da resposta imunológica adquirida contribuem para uma acumulação atrasada de células T CD4+ nos pulmões, causando um agravamento da doença. Neste contexto, demonstramos que a IL-10 induz alterações intrínsecas a estas células, comprometendo a sua capacidade de penetrar o parênquima pulmonar e induzir controlo da infeção. Portanto, decidimos averiguar se níveis elevados de IL-10 teriam impacto na progressão da infeção num hospedeiro com uma resposta T CD4+ previamente estabelecida. Os nossos dados revelaram que a indução da acumulação de células T CD4+ de memória específicas para Ag85 através da vacinação com Bacillus Calmette–Guérin (BCG) preveniu a progressão da doença. Apesar disto, a vacinação não corrigiu a incapacidade das células T CD4+ específicas para o antigénio ESAT-6 de migrarem para o parênquima num ambiente com níveis elevados de IL-10. A diminuição da infiltração de células T no parênquima esteve também associada a um desenvolvimento ineficaz de estruturas linfóides ectópicas. Isto poderá, posteriormente, refletir-se numa insuficiente interação das células T com células mielóides infetadas residentes no granuloma, comprometendo o controlo da infeção. Em suma, estes resultados revelam novos mecanismos através dos quais a IL-10 contribui para a suscetibilidade a Mtb.

Palavras-chave: BCG; Células T CD4+; IL-10; Tuberculose; Vacinação;

ABSTRACT

Regulation of pulmonary CD4+ T cell responses to *Mycobacterium tuberculosis* infection

The COVID-19 pandemic has reversed years of progress in supporting tuberculosis (TB) essential services and in decreasing disease burden. For the first time in more than a decade, TB death rates have increased and are estimated to reach 1.4 million deaths by 2025. Therefore, research breakthroughs are urgent to slow the progression of TB. Control of *Mycobacterium tuberculosis* (Mtb) infection requires the induction of T helper (Th) 1 responses while averting tissue damage. Therefore, understanding the mechanisms governing this delicate balance between protection and immunopathology is of keen significance. Interleukin (IL)-10 is a central player in immune regulation and its expression in Mtb-infected lungs has been described to be necessary for granuloma sterilization. Despite this, IL-10 has also been depicted as a mediator of susceptibility to Mtb infection. However, the precise mechanisms whereby IL-10 plays a detrimental role throughout Mtb infection remain unclear. In this doctoral thesis, I outline the impact of IL-10 overexpression following Mtb challenge in the protective pulmonary CD4+ T cell response. Our data reveal that IL-10 overexpression during chronic infection did not significantly impact the outcome of disease. In contrast, IL-10 overexpression before the onset of acquired immunity contributed for a delayed accumulation of pulmonary Mtb-specific interferon gamma (IFN-γ)-producing CD4+ T cells, which correlated with uncontrolled Mtb proliferation. This delay was found to be caused by intrinsic changes in CD4+ T cells induced by IL-10 that compromised their ability to penetrate the lung parenchyma and induce control of infection. Therefore, we sought to investigate whether IL-10 overexpression would also impact disease progression in a host with a pre-established pulmonary CD4+ T cell response. Our data revealed that Bacillus Calmette–Guérin (BCG)-mediated accumulation of memory Ag85-specific CD4+ T cells in the lungs prevented early disease progression induced by IL-10 following Mtb infection. Despite this, BCG did not restrain the IL-10-mediated impaired accumulation of ESAT-6-specific CD4+ T cells in the lung parenchyma, suggesting that IL-10 impacted the primary immune response in a greater extent than the recall response. The decreased infiltration of ESAT-6-specific CD4+ T cells within the lung parenchyma was correlated with impaired development of ectopic lymphoid structures that are described to allow effective interaction of CD4+ T cells with infected myeloid cells within the granuloma, contributing to the control of Mtb infection. Altogether, the data depicted in this thesis provide significant insights into the mechanisms that govern the detrimental effects of IL-10 during Mtb infection in naïve and vaccinated hosts.

Keywords: BCG; CD4+ T cells; IL-10; Tuberculosis; Vaccination

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LIST OF ABBREVIATIONS

Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
APCs	Antigen presenting cells
B6	C57BL/6J
BALF	Bronchoalveolar lavage fluid
BCG	Bacillus Calmette-Guérin
CCL	C-C Motif Chemokine Ligand
CCR	C-C Motif Chemokine Receptor
CD	Cluster of differentiation
CFP	Culture filtrate protein
CFUs	Colony-forming units
CLR	C-type lectin receptor
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
CXCL	C-X-C Motif Chemokine Ligand
CXCR	C-X-C Motif Chemokine Receptor
CX3CR1	C-X3-C Motif Chemokine Receptor 1
DC	Dendritic Cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin
DMEM	Dulbecco's modified eagle medium
EGFR	Epidermal growth factor receptor
ESAT-6	Early secretory antigenic target 6
FBS	Fetal bovine serum
fDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead transcription factor box 3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
iBALT	Inducible bronchus-associated lymphoid tissue
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGRA	Interferon-gamma release assay
IL	Interleukin

ILC	Innate lymphoid cell
iNOS	Inducible nitric oxide synthase
KLRG1	Killer Cell Lectin Like Receptor G1
ManLAM	Mannosylated lipoarabinomannan
MAIT	Mucosal-associated invariant T
MHC	Major histocompatibility complex
mLN	Mediastinal lymph node
MR	Mannose receptor
Mtb	*Mycobacterium tuberculosis*
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NHP	Non-human primate
NIF	Nodular inflammatory focci
NK	Natural killer
NLR	NOD-like receptor
NO	Nitric oxide
PBS	Phosphate-buffered saline
PBMC	Peripheral Blood Mononuclear Cells
PD-1	Programmed death 1
PPD	Purified protein derivative
PRRs	Pattern-recognition receptors
Rag	Recombinant activating gene
SCID	Severe combined immunodeficiency
SLO	Secondary lymphoid organ
RORγt	Retinoid orphan receptor gamma t
RNS	Reactive nitrogen species
TB	Tuberculosis
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
TST	Tuberculin skin test

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Chapter I

General Introduction

1. Human Tuberculosis: An ancient and evergreen disease

Tuberculosis (TB) has been the leading cause of human death from a single infectious agent. This status has recently changed with the dramatic health threat that COVID-19 pandemic poses to the human population with morbidity and mortality rates higher than those of TB in 2020 (1). Indeed, during the past two years, COVID-19 has fractured and drained health care systems around the globe. Consequently, there has been a significant drop in TB notification rates, which is predicted to have serious consequences for the global control of TB (2). Indeed, an excess of 6.3 million new cases and 1.4 million deaths caused by TB are estimated to occur by 2025 (1–3). Therefore, while progress towards the World Health Organization (WHO) milestone of eliminating TB by 2035 has been hit hard by COVID-19 pandemic (2), it is likely that TB will regain its position as the major killer amongst infectious diseases in a near future. As such, better TB interventions are more necessary than ever before. I hereby outline and discuss the most crucial components of a protective immune response against Mtb and how the scientific community can approach them to improve TB prevention and treatment strategies.

The genus *Mycobacterium* comprises more than 190 species populating a wide range of natural environments (4). These microorganisms are characterized by a thick cell wall mainly composed by a mycolic acid core (5). This lipidic cell wall grants high hydrophobicity and acid-alcohol-fast properties, making mycobacteria weakly gram positive, being usually identified through Zhiel-Neelsen staining (5). The characteristic cell wall of mycobacteria also confers a particular resistance to therapeutic agents and disinfectants, complicating the prevention of disease transmission (5, 6). Some species of mycobacteria have evolved to become more efficient at inducing human and animal TB disease (7). In this regard, three major groups have been considered. The *Mycobacterium tuberculosis* (Mtb) complex group, for instance, includes species with a significant clinical relevance in humans and animals such as Mtb, *M. africanum* and *M. bovis* (8). Nevertheless, the majority of human TB cases are initiated by an infection with Mtb or *M. africanum* (9, 10). In addition, non-tuberculous mycobacteria such as *M. smegmatis*, *M. avium* and *M. ulcerans* are emergent pathogens whose clinical significance varies greatly between species, geographic regions, and the immunological status of the host (11). Contrasting to TB-causing bacteria and non-tuberculous mycobacteria, *M. leprae* and *M. lepromatosis*, the causative agents of leprosy, are also a clinically relevant group of mycobacteria although its incidence has been declining (12).

Mtb has emerged as the leading pathogenic species of mycobacteria for the human host (9, 10). Characterized by its high human-to-human transmissibility via exchange of aerosolized particles, the success of Mtb dwells from its ability to opportunistically colonize the lungs of the host, without causing symptomatic disease (2). Indeed, the capacity of Mtb in generating a productive infection is evidenced by

its longevity. The common ancestor of Mtb was first identified 15000-20000 years ago (13, 14), although it is believed that a progenitor species from which Mtb arose might have been present in East African hominids 3 million years ago (14, 15). It is now appreciated that evolution and human demographic transition resulted in the creation of seven different phylogenetic lineages from human-adapted members of the Mtb complex with distinct characteristics such as virulence or ability to develop antibiotic resistance (9, 16, 17).

TB rampaged the globe for more than a millennium and was the major killer in the capitals of the industrialized world during the nineteenth century (18). Although TB was for a long time considered as an inherited condition, in 1882 Robert Koch discovered that TB was instead caused by a bacterial pathogen (14). Currently, TB persists as a major health concern worldwide, with a quarter of the world's population being estimated to be infected with Mtb (2). Although scientific research is still pursuing improved treatments and effective vaccines, TB is still a long way from being a disease from the past.

1.1. Disease burden worldwide

Globally, the highest number of TB cases occurs in Asia and Africa, with only 3% of the total number of cases occurring in the European region (2). However, the COVID-19 pandemic had a major effect in the notification rates, particularly in the developing countries. Strikingly, the reported numbers of people newly diagnosed with TB fell from 7.1 million in 2019 to 5.8 million in 2020 (2, 19). Sixteen countries account for 93% of this reduction, with India, Indonesia and the Philippines being the countries with the highest contribution for this reduction (2). Additionally, COVID-19 pandemic has reduced the access to diagnostics and treatments. Indeed, in 2020, the total investment on TB diagnosis, treatment and prevention was estimated to have been less than half of what was needed in 2020 (2). As a result, the frequency of provided TB preventive treatments decreased about 21%, and the numbers of TB patients undergoing treatment for drug-resistant TB were estimated to be only 1 out of 3 of those in need (2). The reduced access to treatment likely contributed for the increase in TB death rates for the first time in more than a decade, with 1.5 million deaths in 2020 (2). Globally, about 85% of these deaths occurred in the African and South-East Asia regions, with India alone accounting for 34% of total TB deaths. Among Human Immunodeficiency Virus (HIV)-negative people, 16% of TB deaths were children with less than 15 years old (2).

The most urgent actions to mitigate and reverse the impact of the COVID-19 pandemic on TB are to restore access to essential TB services. This progress requires adequate funding for TB diagnostic, treatment, and prevention services, sustained over many years. However, with 98% of reported TB cases falling in low- and middle-income countries, funding falls short than what is needed.

1.2. The spectrum of *M. tuberculosis* infections and their diagnosis

The inhalation of infected aerosol droplets can originate a set of different outcomes which are distinguished by the type of immune response initiated by the host. When an infection overcomes early physical and immunological defences, the host engenders an acquired immune response. This acquired immune response relies on the expansion of antigen-specific cells to combat the pathogen and, ultimately, give rise to immunological memory. In this regard, tuberculin skin tests (TSTs) and interferon (IFN)-γ release assays (IGRAs) measure immune sensitization against Mtb and reflect whether an Mtb-specific acquired immune response has occurred (20, 21). These tests are the only diagnostic tool used to detect subclinical Mtb infection in humans. On the one hand, IGRA measures the cytokine IFN-γ upon stimulation of whole blood samples with the Mtb antigens Early Secretory Antigenic Target (ESAT)-6, Culture Filtrate Protein (CFP)-10, and TB7.7. As these antigens were deleted from the genome of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and are absent in most environmental mycobacteria (22, 23), IGRA tests allow TB diagnosis in BCG vaccinated people. On the other hand, TST measures delayed type hypersensitivity caused by intradermal administration of a crude mixture of mycobacterial antigens called purified protein derivative (PPD) (21, 23). The limitations of TST- and IGRA-based diagnosis will be discussed below.

Epidemiological and genetic studies support the hypothesis that humans can eradicate Mtb immediately following exposure, without the development of specific immune responses. Reports studying this early clearance of Mtb have relied on highly exposed individuals from professional or household settings to infer on the resistance of an individual to develop an established infection based on the performance of TST and/or IGRA tests throughout time. These studies reveal that a proportion of heavily exposed individuals remain TST negative, and do not become infected (24, 25). As an example, of more than seven hundred household contacts of 130 sputum smear-positive TB cases in Gambia, only 41% tested positive for PPD skin test, and 30% were tested positive for IGRA assay (26). These findings suggest that some individuals mount an innate immune response that is very effective in eliminating the infection before the acquired immune response is initiated. Although these studies define exposure by a function of duration, proximity, and grade of sputum positivity of the transmitter, it remains unproved that exposed individuals did, in fact, interact with the bacteria. This caveat prevents the identification of the very initial events that potentially lead to the early clearance of Mtb. The use of animal models of Mtb infection are a rational and forthcoming approach to this question given that it allows the assessment of detectable colony-forming units (CFU) in the lungs immediately after exposure with the pathogen. Studies with the rabbit model of Mtb infection revealed that, although all rabbits exposed to a very low dose (10-50 CFUs)

of HN878 Mtb strain become positive for TST test and present detectable bacteria in the lungs three hours post-exposure, only 88% have detectable CFUs 4 weeks following infection (27). These results support the hypothesis derived from human data that early clearance of Mtb infection is a possible outcome of disease. Interestingly, when the initial dose of infection increases, all rabbits display detectable CFUs 4 weeks following infection (27). These findings suggest that the dose of transmission is a limitation for the early clearance of the bacteria by the host.

In addition to previous conclusions, these findings also indicate that the initial dose of infection is a differential factor between human transmission and animal experimental models of TB. Indeed, the standard mouse model of TB relies on animals infected with an aerosolized dose of 50–100 CFUs, whereas different evidence suggest that the typical human infection occurs upon the transmission of only 1-3 bacilli (28, 29). Studies using ultra-low doses of inoculum in mouse models are just now starting. A recent study revealed that mice infected with an ultra-low dose of Mtb better resembled human disease, presenting more heterogenous bacterial burdens and pathology (30). In experiments using nonhuman primate (NHP) models of infection, the ultra-low dose inoculum (<30 CFUs) was also shown to be reproducible (31). These findings point out that there is a correlation between the infectious dose and the degree of susceptibility to Mtb infection in experimental animal models (30, 32–34). Accordingly, the magnitude of the bacterial doses that humans receive is also believed to dictate outcome of disease. One of the most striking examples is the Lubeck disaster that occurred in Germany in 1929. After the administration of a BCG inoculum contaminated with a virulent strain of Mtb, 29% of the inoculated children died and 68% developed clinical signs of infection (35). These numbers are much higher than the frequency of symptomatic individuals and mortality rate observed in individuals infected through the normal route. Although the quantification of Mtb contamination was not precise, the infectious dose was likely a key determinant for the outcome observed. Despite this, the very early host innate immune mechanisms against Mtb infection remain largely unknown. Understanding the factors that determine clearance or development of an established infection is essential to pinpoint this issue, as the lack of defined immunological correlates of protection for TB is the major constraint for vaccine development.

When the initial host immune response is not sufficient to prevent bacterial expansion, the onset of the acquired immune response is triggered by the host. Most frequently, infected individuals develop a commensal-like relationship with the bacteria and control the infection into a latency state (called latent TB) (36, 37). Latent TB does not present clinical signs of disease progression, as latent TB patients typically present normal chest X-Rays and negative sputum tests (36, 37). Therefore, latent TB is often identified by positive TST or IGRA tests (38). During latent TB, a robust host immune response allows the

containment of infection. However, 5-10% of screen-test-positive patients show a disruption of latency into active disease, usually referred to as disease reactivation (37). Active TB is characterized by the presence of clinical symptoms including fever and cough, and in disease transmission (39). The development of active TB is frequently coupled with a compromised host immune response (39). Accordingly, the highest risk factor for TB reactivation is HIV co-infection (2). In addition, undernutrition, alcohol use disorders, smoking and diabetes are among the most relevant risk factors for the development of active TB (2, 39). The specific immunological factors influencing disease reactivation will be discussed in the following sections of this chapter. Importantly, since TST and IGRA tests measure the presence of memory T cells against Mtb, and these responses can persist for years even after the infection has been eliminated, active TB patients also present positive IGRA and TST tests (Figure 1) (40). Therefore, there is no gold standard test for TB infection as immunological tests do not discriminate between active, latent, or resolved TB infection (40). Despite this, other resources are available for the confirmation of active TB disease. For instance, the detection of viable bacteria in bronchoalveolar lavage fluid (BALF) and radiography screening are also used to aid the detection of pulmonary TB disease. It is important to highlight, however, that individuals with clinical signs of disease present heterogeneous lung pathological representations from caseous necrosis hypoxic lesions to liquefied cavities (41). Despite this, TB is commonly depicted as a binary distribution of active vs latent infection. This means that the historical division between latent and active disease is likely underappreciating the complexity of host-Mtb interactions, which could be affecting the development of novel preventive and therapeutic approaches.

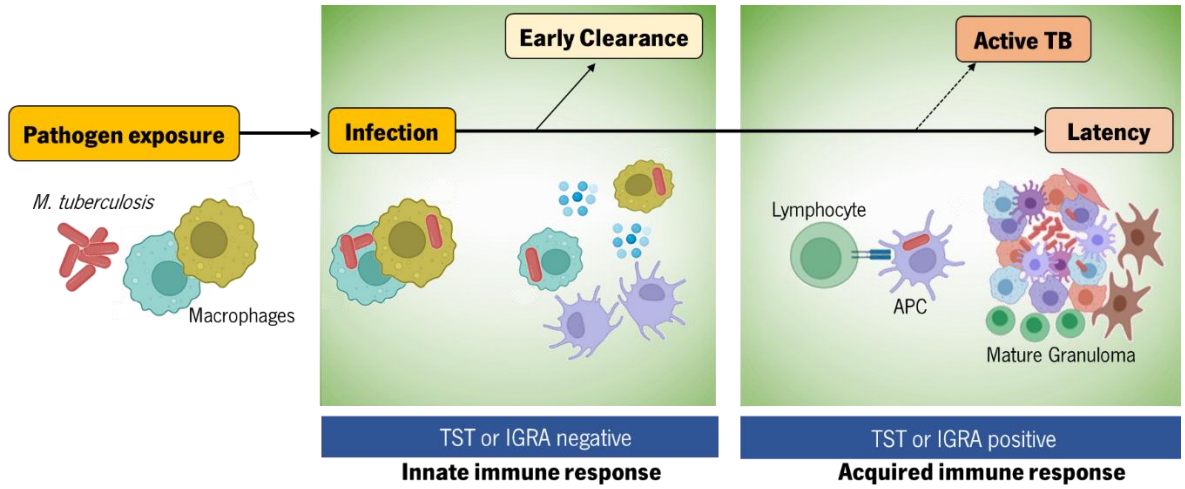

Figure 1: Distinct outcomes of *M. tuberculosis* infection. After aerosol exposure, Mtb is phagocytosed by cells present within the lower respiratory airways. Infection with Mtb leads to the onset of the innate immune response. Early clearance of Mtb occurs when the innate immune response is effective at eliminating the bacteria without requiring the onset of the acquired immune response. If an acquired immune response is developed, the individual reacts positive for TST and IGRA tests and most frequently enters latency. Immunocompromised individuals can experience disease reactivation into symptomatic and transmissible disease (active TB). APC: Antigen-presenting cell; TST: tuberculin skin tests; IGRA: IFN-γ release assays.

1.3. BCG vaccination

Vaccination offers the most sustainable and cost-effective answer for the longstanding control of an infectious disease (42, 43). Vaccines are designed to induce humoral and cellular responses against specific pathogens and this way promote rapid control of an infection. Precisely 100 years after its first administration to a newborn, BCG remains as the only licensed vaccine used to prevent TB. BCG is a live attenuated vaccine developed form of the cattle pathogen *M. bovis* which after almost 100 years of use has been estimated to have saved the lives of millions of people (44). Although BCG has been shown to have a 60-80% protective efficacy against severe forms of disseminated TB in children (45–48), its efficacy against adult pulmonary TB appears to be highly variable (49). The duration of the protection conferred by BCG is also a controversial subject. Although some studies suggest that BCG provides long-term protection against TB (50), others reveal a limited effect when infection occurs long after vaccination (51–53). So far, a precise explanation as to why BCG vaccination leads to such disparity in protection has not been identified; however, it is likely a multi-factorial situation.

The worldwide use of BCG for the purpose of vaccination against TB encouraged each country to maintain its own supply, originating multiple substrains of BCG. The genomic alterations arisen from persistent subcultivations of the original strain have been advocated as a factor leading to antigenic variation between substrains from distinct geographical regions (43, 45, 54, 55). Accordingly, experimental studies revealed that vaccinated mice generate differential immune responses and resistance against infection, depending on the BCG substrain (56, 57). In humans, a randomized controlled trial showed that there were significant differences in the immune response triggered by the different BCG substrains in newborn infants (58).

Another factor that might be contributing for confounding BCG effectiveness is the pre-sensitization with environmental mycobacteria. Mice sensitized with multiple environmental mycobacteria prior BCG vaccination, raise an immune response that restricts vaccine-induced immune responses and culminates with decreased protection against Mtb infection (59). In humans, greater BCG efficacy is observed in latitudes further away from the equator (60). This finding is consistent with greater exposure to environmental mycobacteria present in the warm and wet climates closer to the equator. One study compared mycobacteria sensitization and the generation of vaccine-induced immune responses following BCG administration. This study revealed that UK adolescents did not show sensitization to mycobacteria, as determined by negative TST tests, prior BCG administration, and presented an 80% increase in the generation of IFN-γ-dependent immune responses following vaccination. On the other hand,

approximately 60% of Malawi adolescents displayed positive TST responses before vaccination, and the generation of vaccine-elicited IFN-γ-dependent immune responses was scarce (61).

The strategy used for BCG vaccination is likely the most relevant factor contributing for its efficacy. For instance, the timing for BCG administration could be determinant. It has been shown that delaying BCG vaccination much longer after birth results in decreased immunogenicity, with the generation of lower frequencies of BCG-specific IFN-γ-producing T cells when comparing with infants vaccinated soon after birth (62–64). Additionally, the route of BCG administration is, currently, a central matter of discussion. Although BCG was originally intended for oral administration in children, nowadays it is delivered most often by the intradermal route. Nevertheless, recent evidence in animal models suggest that mucosal administration could be a more efficient approach (65–67). In strike contrast, a recent study testing BCG administration through the intravenous route revealed that intravenous administration of BCG prevents TB in NHP (68).

TB can be cured effectively with drug treatment. However, increasing incidences of drug resistance render therapeutic options as insufficient. Therefore, a combination of better drugs, diagnostics and vaccines should improve TB control (2). BCG not only confers reliable protection against disseminated disease in children, but has also been shown to have beneficial heterologous effects outside the target disease (69). Therefore, it is reasonable to prioritize the search for approaches to improve BCG. To achieve this goal, it is urgent to understand what is failing during the onset of the immune response elicited by BCG. As discussed above, genomic alterations in BCG batches from different countries influence protection against Mtb. Therefore, a more efficient method for BCG inoculum maintenance should be explored. The pre-exposure with mycobacteria may highlight interesting mechanisms contributing for the disruption of BCG-mediated protection, although it remains unreasonable to discard the premise that mycobacteria sensitization is instead masking BCG protectiveness. Currently, the strategy used for BCG vaccination, whether the time or route of administration, is a priority, and most studies are dedicated to mechanistically dissect how they contribute for the outcome of subsequent Mtb infection. The conclusions arisen from these studies have been put in the pursuit for adjuvants in BCG vaccination (70). Nevertheless, most vaccines reaching clinical trials have demonstrated poor effectiveness. Thus, the need to unravel novel vaccination strategies against TB remains a central problem within the health research field.

2. Host immune response against *M. tuberculosis*

The typical host immune response against Mtb infection relies on a cascade of multifaceted cellular and molecular events that include the release of pro-inflammatory cytokines that influence the onset of the pathogen-specific immune response. The innate and acquired immune responses act in combination to mount a protective immune response in which the growth of the pathogen is restricted without pathological consequences to the host. As discussed below, when innate immune cells are not capable of resolving the infection, the acquired immune response will intervene and enhance these responses. Therefore, understanding the interconnections between the two branches of the host immune system is vital to unravel potential targets for preventive and therapeutic approaches.

2.1. Innate Immune Responses

As described above, there are compelling evidence that innate immune responses can contribute significantly to TB resistance. Immunity against Mtb begins when pathogen-associated molecular patterns (PAMPs) located at the surface of Mtb are recognized by pattern recognition receptors (PRRs) present at the surface of innate immune cells. PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), mannose receptors (MRs), and dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (71–73). Upon recognition, innate immune cells uptake the bacteria and initiate antimicrobial effector functions to contain bacterial growth. Indeed, upon exposure, alveolar macrophages are the first immune cells to interact and uptake Mtb. Over the course of infection, Mtb is also phagocytosed by other cell types within the lung parenchyma, mostly recruited monocyte-derived macrophages. In fact, macrophages remain the primary cellular reservoir for Mtb throughout the infection (74). Upon interaction with Mtb, macrophages initiate a network of signalling pathways that lead to a multiplicity of distinct gene expression profiles. One of the most well-studied examples is the activation of nuclear factor kappa light chain enhancer of activated B cells (NF-kB) pathway after the engagement of macrophage TLR2 with mannosylated lipoarabinomannans (ManLAM) from Mtb. Importantly, NF-kB activation triggers the initiation of several direct and indirect defence mechanisms. For instance, the generation of reactive nitrogen species (RNS) is a process known to be essential for the inhibition of bacterial replication inside the macrophage and other infected cells.

The formation of RNS from L-arginine by the inducible nitric oxide synthase (iNOS) is considered a key first line defence against Mtb. Indeed, infection with Mtb increases the expression levels of inducible iNOS in humans and mice (75–77). Accordingly, drug-mediated inhibition of iNOS activity and the genetic deficiency of iNOS results in increased lung bacterial proliferation and dissemination (78–81). The key

role of iNOS in the control of Mtb infection resides in the bactericidal capacity of nitric oxide (NO) and other RNS residing within the phagosome. In addition, NO was found to be a relevant signalling molecule. Mtb-infected murine macrophages deficient for iNOS and stimulated with IFN-γ were found to suppress NF-kB signalling, preventing hyperinflammatory responses that could be harmful to the host (77). Although macrophages are armed with a plethora of antimicrobial mechanisms, the efficient neutralization of intracellular Mtb, including through iNOS expression, is largely dependent on direct and indirect signals from IFN-γ-producing $CD4^+$ T cells (82, 83), although earlier during infection innate cellular sources of IFN-γ are also crucial (84, 85). In fact, IFN-γ is well described as a critical inducer of macrophage antimicrobial mechanisms (82, 83). In addition to the aforementioned microbicidal mechanism, macrophages are also capable of activating death pathways to prevent further intracellular replication (86). Apoptosis is a highly regulated and controlled process that maintains the integrity of the plasma membrane, enabling Mtb growth restriction (87). Death by apoptosis minimizes inflammation and pathology by containing the dismembered cellular contents within membrane-bound vesicles, called apoptotic bodies (87). These apoptotic bodies emit signals that allow their digestions by neighbouring phagocytes. In accordance with the beneficial impact of apoptosis in Mtb-infected macrophages are studies revealing that Mtb exploits apoptosis-promoting signalling pathways to block host macrophage apoptosis, favouring its proliferation (88). On the other hand, necrosis is a traumatic cell lysis event that enables pathogen release and spread to the adjacent cells. The harmful consequences of necrosis induction during Mtb infection will be discussed in section 3.1 of this chapter.

In addition to direct microbicidal mechanisms, activated macrophages also produce signalling molecules that recruit and activate other innate immune cells. Cytokines such as Interleukin (IL)-12, tumour necrosis factor (TNF), IL-6, and IL-1 play a key role in the innate inflammatory response but are also pivotal for the onset of the acquired immune response. In this regard, IL-12 is a cytokine predominantly produced by macrophages and dendritic cells (DCs) and is formed by the covalent bond formed between IL-12p40 and IL-12p35 subunits. The lack of IL-12p40 subunit and of its receptor subunit, IL-12Rβ1, leads to increased Mtb growth and mortality (89–93). As the importance of IL-12 signalling during Mtb infection is tightly associated with the development of the acquired immune response, its relevance in the context of Mtb infection will be discussed in the next section. It is relevant to highlight, however, that IL-12 production is essential for the subsequent generation of a pro-inflammatory microenvironment that contributes for the control of bacterial growth. Indeed, macrophages stimulated under an anti-inflammatory microenvironment undergo an alternative activation process characterized by increased production of anti-inflammatory mediators, thereby engaging in tissue repair

functions instead of activating bactericidal pathways (94, 95). Particularly, alternatively activated macrophages increase arginase 1 expression, an enzyme that competes with iNOS for the common substrate, l-arginine. Therefore, L-arginine metabolism in alternatively activated macrophages is driven towards the production of proline, the building block of collagen, instead of NO and citrulline (95). Consequently, alternatively activated macrophages are less able to restrict disease progression and represent a permissive niche for the crescent granuloma (96, 97). Although pro-inflammatory cytokine production is essential to ensure classical macrophage activation and optimize the stimulation of bactericidal mechanisms, host-intrinsic programming also contributes for the segregation of macrophage activation. Indeed, macrophages with embryonic origin, such as alveolar macrophages, present a phenotypic resemblance to alternative activated macrophages and are more permissive to Mtb infection. In contrast, monocyte-derived interstitial macrophages present a more pro-inflammatory profile and are more fit to restrict Mtb growth (74).

In addition to the aforementioned cytokines, macrophages are also important producers of a vast array of chemokines such as C-X-C Motif Chemokine Ligand (CXCL)1, CXCL2, C-C Motif Chemokine Ligand (CCL)5, CXCL8, CXCL9 and CXCL10 that are fundamental for the recruitment of other innate and acquired immune cells, contributing for granuloma development (98, 99). Particularly, DC accumulation in the infected tissue is fundamental for protective immunity against Mtb infection. DCs are considered professional antigen presenting cells (APCs) and play a fundamental role in bridging innate and acquired immune responses. Upon infection, DCs process antigen and increase surface expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules CD80/CD86 to prepare for antigen presentation. DCs migrate through the lymphatic system into the mediastinal lymph nodes (mLN) in a process dependent on IL-12p40 and the C-C Motif Chemokine Receptor (CCR) 7 (the receptor for homeostatic chemokines CCL19 and 21). Within the mLN, antigen-carrying APCs engage via MHC-II or MHC-I with naive $CD4^+$ and $CD8^+$ T cells, respectively, that will, in turn, become activated and mount antigen specific responses. The timeframe between Mtb arrival to the airways and antigen presentation and priming of naive T cells in the mLN is believed to be a factor contributing for poor control of the infection. In fact, patients carrying a specific mutation associated with decreased numbers of conventional DCs exhibit increased susceptibility to mycobacterial infections (100). This subject will be explored on section 2.2.1 of this chapter.

As mentioned above, the inflammatory process elicited by macrophages and DCs during infection also triggers the accumulation of other innate immune cells to further promote inflammation and tissue remodelling. Specifically, the recruitment of neutrophils is key in the control of infection. Neutrophil

antimicrobial mechanisms include the production of ROS and RNS, and secretion of antimicrobial enzymes including a-defensins, matrix metalloproteases, and lipocalin that can assist in tissue repair, restrict Mtb growth and promote apoptosis of infected cells (101). Thus, neutrophils play a protective role in early stages of Mtb infection. Indeed, neutrophil depletion prior or throughout the first days of Mtb infection associates with increased lung bacterial proliferation (102). One of the factors that might contribute to the protective function of neutrophils is their ability to produce IL-12, TNF-α and IL-1β. These cytokines are essential to expand the pro-inflammatory signals and assist T cell activation. On the other hand, neutrophils are also producers of IL-10 (103), an anti-inflammatory cytokine that is essential to balance pro- and anti-inflammatory responses. Indeed, despite their protective role early during the infection, neutrophils are also depicted as the main contributors of immunopathology in Mtb-infected hosts. In fact, neutrophils are one of the most prevalent immune cell population present in BALF and sputum samples of active pulmonary TB patients, second to lymphocytes (104, 105). In animal models, neutrophils are also associated with susceptible phenotypes and their exacerbated accumulation either during early or chronic stages of the disease has been associated with poor prognosis (106–110). Therefore, there is an association between neutrophilia and the development of aggressive forms of TB disease that will be further discussed in section 3.1 of this chapter. Importantly, neutrophil recruitment can be potentiated through IL-17 production (111). In fact, IL-17 produced within 4-8 hours post-infection was demonstrated to increase neutrophil recruitment by promoting IL-6, granulocyte colony-stimulating factor (G-CSF) and CXCL8 production. Similar to the role of neutrophils in immunity against TB, IL-17 also plays a controversial role during Mtb infection, as will be discussed in the next section.

Other innate immune cells present in the lungs of mice and humans have emerged as relevant sources of pro-inflammatory cytokines as well as key regulators of protective immunity against Mtb. Unconventional T cells include have been increasingly depicted as highly relevant innate immune cells. For instance, innate lymphoid cells (ILCs) are a group of heterogeneous innate immune cells that belong to the lymphoid lineage but do not express recombinant activating gene (RAG) and depend on the γc component of the IL-2 family of receptors (112). With the exception of NK cells, ILCs are tissue-resident cells and populate most frequently mucosal barrier surfaces (113). As a functional and phenotypical heterogeneous population, these cells were classified into three different clusters: group 1 that produce IFN-γ and include natural killer (NK) cells and ILC1s; group 2 (ILC2s) that produce IL-5 and IL-13; and group 3 (ILC3s) that produce IL-17 and IL-22. As early sources of IFN-γ, Group 1 ILCs are relevant for the rapid control of Mtb growth by stimulating macrophage antimicrobial mechanisms. On the other hand, ILC3s have been recently described to participate in the generation of protective immune responses in

the context of Mtb infection and in inducing vaccine-mediated protection (114). This topic will be further explored on section 3.2 of this chapter.

γδ T cells are also a cluster of unconventional $CD3^+$ T cells that have a T-cell receptor arranged with a γ and a δ chain. Upon recruitment to the infection site, these cells produce IL-17 in response to IL-23 and exert cytotoxic effector functions (118). This innate cell population was shown to dominate early production of IL-17 during Mtb infection in mice, and to remain as major producers of this cytokine throughout infection (115). Accordingly, the adoptive transfer of γδ T cells in Mtb-infected NHP limited disease to the infected lobe and decreased bacterial loads in the lungs (116). In TB patients, however, IL-17 was found to be produced mostly by conventional T cells, although γδ T cells and Mucosal-associated invariant T (MAIT) cells were also found to be relevant contributors (117). γδ T cells also emerge as important players in BCG-mediated protection. Indeed, BCG vaccination in NHP was reported to expand γδ T cells that supported Mtb growth restriction via perforin and granulysin production (118). Despite this, further studies are necessary to understand the precise roles these cells can have in the control of Mtb infection.

MAIT cells and NKT cells constitute a subset of T cells that recognize non-peptidic antigens. In fact, MAIT and NKT cells recognize pathogen metabolites and lipids presented by CD1, and MHC-related protein 1 (MR1), respectively. Current literature does not clearly identify a key role for NKT cells in human TB. Despite this, their fast activation and distinctive functions can potentially poise this cell population as an interesting player in the context of Mtb infection (119). MAIT cells, on the other hand, contribute to the destruction of infected cells and activation of other immune cell types via release of perforin and granzymes. MAIT cells are also important sources of TNF-α, IFN-γ, and IL-17A. In a recent study, MAIT cells were shown to have a dual role during Mtb infection in mice. Indeed, MR1-defficent mice display similar survival rates as wild-type (WT) mice. Furthermore, vaccination with microbial riboflavin-derived antigens improves MAIT cell proliferation but delays $CD4^+$ T cell priming, suggesting that MAIT cells may contribute for the delayed onset of the acquired immune response. Nevertheless, treating Mtb-infected mice with microbial riboflavin-derived antigens during the chronic stage reduced lung bacterial burdens (120). Interestingly, during active TB disease in humans, the frequencies of MAIT cells in circulation and in the lungs decrease when comparing with latently infected individuals (119, 121, 122).

Despite the unique capacities of unconventional innate immune cell populations, their role in the Mtb-induced immune response is not completely recognised. It seems likely that the contribution of each one of these cellular populations is overshadowed by the activity of acquired immune cells. Despite this,

it is undeniable that the onset of the innate immune response is key for the immediate containment of Mtb growth, and for the onset of the acquired immune response.

2.2. Acquired Immune Response: $CD4^+$ T cells as the cornerstone of host immunity

Host resistance against Mtb infection relies on the combined efforts of the innate and acquired immune responses. Despite this, while the capacity of the innate immune response to contribute for the resolution of Mtb infection remains difficult to address, the fundamental role of the acquired immune response in controlling an established infection and in providing better protection against secondary encounters is unquestionable (123–126). Indeed, the mouse model of TB disease was essential to demonstrate that the timeframe for the control of bacterial growth overlaps with the onset of the acquired immune response (125, 127). In humans, the importance of the acquired immune response for the control of TB is demonstrated by the evident susceptibility of individuals with an impaired acquired immune response. Particularly, Severe combined Immunodeficiency (SCID) children and individuals infected with HIV display impaired acquired immunity and are highly susceptible to Mtb infection being unable to restrict Mtb growth in the lungs (123, 128).

The acquired immune response comprises the participation of T and B cells. The role of B cells during Mtb infection has been, for the most part, neglected. B cell functions considerably rely on antibody production. Nevertheless, the relevance of antibody-mediated immunity against Mtb is still questioned. Although granulomata from NHP were shown to be surrounded by B cell clusters that secrete Mtb-specific antibodies (129), the depletion of B cells using therapeutic monoclonal antibody rituximab has heterogenous effects (130). However, administration of monoclonal antibodies raised against Mtb antigens or pooled antibodies from mice or humans were shown to ameliorate disease in animal models (131–133). These findings urged the search for antibody-mediated protective immunity against TB. Interestingly, a recent study revealed that intravenous administration of BCG induces robust Immunoglobulin (Ig) M responses that associate with prevention of Mtb infection in NHP (134). Therefore, current progresses highlight a potential importance of antibody responses as markers and mediators of protection against TB. Despite this, the overall contribution for B cells in the outcome of Mtb infection remains to be determined. In addition to producing antibodies, B cells are also competent APCs and are a source of various important cytokines (135, 136). Therefore, B cells are also key regulators of T cell responses (135, 136). Recent studies suggest an association between B cell accumulation in the

granuloma and improved protection against infection. This subject will be discussed in more detail in section 3 of this chapter.

While the role of B cells is still being defined, the role of T cells in the immune response against Mtb is unarguably critical to the control of infection. As described above, upon antigen recognition, APCs carrying Mtb antigens migrate from the lungs into the mLN, where primming and differentiation of naive $CD4^+$ and $CD8^+$ T cells occurs. As naive T cells become activated, they proliferate, upregulate CD69 and CD44, and downregulate CD62L (137). This process occurs between days 9-14 post-infection in the mouse model of infection (Figure 2) (125). The activation of $CD4^+$ and $CD8^+$ T cells is also accompanied by the upregulation of chemokine receptors, specifically CXCR3 and CCR5 (138, 139). The expression of these chemokine receptors is essential for their subsequent migration, through the bloodstream, to the infected lungs in response to their ligands (CXCL9, 10, and 11 for CXCR3) and (CCL3, 4 and 5 for CCR5) (138, 140, 141). In the infected tissue, these T cell populations will actively fight Mtb progression through cytolytic activity and cytokine production.

Although Mtb infection elicits both $CD8^+$ and $CD4^+$ T cell responses, evidence for an essential role of $CD8^+$ T cells is not as compelling as for $CD4^+$ T cells. Indeed, the increased risk of TB in HIV-infected individuals demonstrates the crucial role of $CD4^+$ over $CD8^+$ T cells (128). Additionally, mice infected with Mtb survive longer in the absence of $CD8^+$ T cells than in the absence of $CD4^+$ T cells, which have markedly low survival rates (142). Similarly, $CD8^+$ T cell neutralization three weeks after infection does not impact the survival of C57BL/6 (B6) mice (143). Therefore, although Mtb-infected mice without $CD8^+$ T cells present a reduced capacity to control lung Mtb growth (144), their role in the control of Mtb infection appears to be limited.

The reasoning for the small contribution of $CD8^+$ T cells for host immunity against Mtb is still debatable. Initially, the availability of Mtb peptides for loading into MHC-I was believed to be limited as Mtb locates preferentially in the phagosomes of infected macrophages. However, it is now recognized that Mtb antigens can access the cytosol and be processed both in by endocytic and cytosolic pathways. These data show that Mtb antigens are loaded in both MHC-I and MHC-II (145). Accordingly, the Mtb antigen TB10.4 elicits immunodominant $CD8^+$ T responses in in both mice and humans, with one third of the total activated $CD8^+$ T cells being TB10.4 specific in B6 mice lungs (146). However, it has recently been shown that while $CD4^+$ T cells that recognize Mtb antigens, specifically Ag85b and ESAT-6, are able to engage with Mtb-infected macrophages and inhibit Mtb growth *in vitro*, TB10.4-specific $CD8^+$ T cells do not recognize infected macrophages or prevent bacterial growth (147). Importantly, it has been suggested that viable bacteria might use TB10.4 as a decoy antigen and that this TB10.4-specific $CD8^+$ T cell

responses is overshadowing subdominant $CD8^+$ T cell responses that can recognize infected macrophages. Furthermore, the effector functions of $CD8^+$ T cells in the context of Mtb infection, has been shown to depend greatly on $CD4^+$ T cells. For instance, IFN-γ production by lung effector $CD8^+$ T cells is compromised in the absence of $CD4^+$ T cells (148–150), likely because $CD4^+$ T cells prevent exhaustion of the $CD8^+$ T cell response during Mtb infection (150).

The evidence for the key role of $CD4^+$ T cells in the control of Mtb infection is striking both in animal models and in humans (123–126). In mice, the control of Mtb proliferation overlaps with the peak of cytokine-producing Mtb-specific $CD4^+$ T cells accumulation, approximately 20 days after infection (125) (Figure 2). Importantly, the genetic ablation or antibody-mediated depletion of $CD4^+$ T cells results in uncontrolled Mtb proliferation after the first 20 days of infection (151–153), and with mice succumbing to infection much earlier than control animals. In humans, the role of $CD4^+$ T cells is evident in AIDS patients, who have lower $CD4^+$ T cell numbers than healthy individuals, and for this reason are highly susceptible to the development of primary Mtb infection, reinfection, and reactivation (128). In fact, in HIV^+ individuals that positively respond against purified protein derivative (PPD) stimulation have 8-10% annual risk of developing active tuberculosis, contrasting to the 10% lifetime risk of PPD^+HIV^- individuals (154–156). These findings clearly demonstrate that while other immune players may take part in protection against Mtb infection, they alone cannot compensate for the lack of a $CD4^+$ T cell response (153, 157, 158).

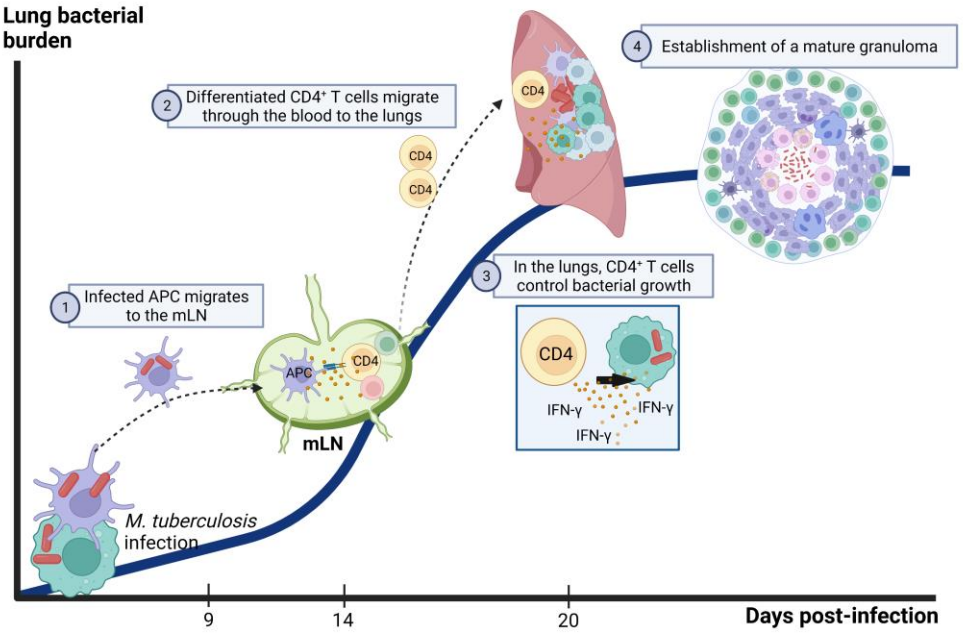

Figure 2: Control of *M. tuberculosis* infection coincides with the peak of the accumulation of pulmonary IFN-γ-producing CD4⁺ T cells. In the mouse model of Mtb infection, Mtb is phagocytosed by patrolling cells resident in the lower respiratory airways. Infected APCs migrate via lymph into the mLN after approximately 9 days post-infection **(1)**. Within the mLN, naive CD4⁺ T cells recognize Mtb antigens via interaction with APC and initiate priming and differentiation. Effector CD4⁺T cells migrate through the blood vasculature to the lung parenchyma in response to inflammation **(2)**. The peak of the accumulation of these cells, approximately 20 days after infection, coincides with the stabilization of lung bacterial burdens. In the lungs, antigen-specific IFN-γ-producing CD4⁺ T cells direct directly and by the production of cytokines with infected myeloid cells to allow control intracellular Mtb growth **(3)**. The accumulation of effector T cells initiates the formation of the mature granuloma **(4)**. APC: Antigen-presenting cells; mLN: mediastinal lymph nodes. Created with BioRender.com.

2.2.1. Th1 cells

Naive CD4⁺ T cells can polarize into different phenotypic and functional subsets, depending on their surrounding microenvironment. The immune response against Mtb is predominantly driven by T helper (Th) 1 cells which are dependent mostly on IL-12 signaling and is controlled by the expression of the transcription factor T-bet. More importantly, CD4⁺ T cells with a Th1 phenotype have a traditional predominant IFN-γ production, which is essential for protection against intracellular pathogens such as Mtb. Mice deficient on IFN-γ present uncontrolled bacterial proliferation, widespread lung tissue necrosis, disseminated infection and rapidly succumb to Mtb infection if not treated with exogenous recombinant IFN-γ (153, 159, 160). A similar phenotype was observed in mice subjected to IL-12 neutralization (89, 161). Not surprisingly, the vital role of IL-12 signaling for the resistance against Mtb infection is dependent on IFN-γ as demonstrated by studies showing that IL-12 administration to IFN-γ deficient animals did not confer protection against Mtb infection (162). Along with the data from the mouse model, humans with inborn errors in the IL-12/IFN-γ axis were shown to have a predisposition for the development of clinical

TB disease (163, 164), and individuals that develop autoantibodies against IFN-γ tend to present disseminated TB disease (165). One of the best described mechanisms of IFN-γ is the activation of the macrophage antimycobacterial defense mechanisms. IFN-γ-activated macrophages are better able to eliminate intracellular pathogens through iNOS expression and RNS production, a process detailed in the previous section. In addition, IFN-γ production has also been shown to contribute for an improved phagosome-lysosome fusion (166, 167), and activate autophagic pathways (168). Accordingly, IFNγ-deficient mice present a defective production of RNS (159, 160). Besides the IFN-γ direct role in activating macrophages, this cytokine also plays important roles in limiting inflammation, namely by restricting neutrophil recruitment through the suppression of Th17 responses.

Although $CD4^+$ T cells are the main producers of IFN-γ during Mtb infection, other lung immune cells in may contribute with IFN-γ production. In fact, mice deficient in $CD4^+$ T cells only display a transient impairment in IFN-γ production, that is overcome after the first month of infection (153). Interestingly, the stabilization of IFN-γ levels did not protect mice from exacerbated infection. Although the delayed IFN-γ production likely accounts for the increased susceptibility of these mice, these data support the crucial role of IFN-γ-dependent and -independent $CD4^+$ T cell mechanisms during Mtb infection. In accordance with this premise is the observation that mice replenished with IFN-γ-deficient $CD4^+$ T cells are not capable of maintaining long-term control of Mtb infection (149). Although the IFN-γ-independent mechanisms of $CD4^+$ T cell protection remain unclear, the ability of lung $CD4^+$ T cells to interact with infected phagocytes is an important factor (169, 170). Altogether, these data suggest that $CD4^+$ T cell derived IFN-γ is essential for protective immunity against Mtb infection. Nevertheless, IFN-γ production is not a correlate of protection as it has been demonstrated that the presence of this cytokine does not correlate with improved protection. Thus, other protective features associated with this population must be explored. This subject will be discussed on section 2.2.1 of this chapter.

In addition to IFN-γ, Th1 cells also produce TNF-α, a potent modulator of early inflammatory responses but can also enhance the IFN-γ-dependent mechanisms. Indeed, TNF blockers have been shown to reduce IFN-γ-mediated mechanisms and induce apoptosis of several immune cells, including $CD4^+$ T cells, monocytes and $CD8^+$ T cells (171). Furthermore, TNF supports anti-Mtb immunity by promoting the production of chemokines (172), up-regulating adhesion molecules (173) and inducing apoptosis of infected macrophages (174). For these reasons, Mtb-infected mice under TNF-α-neutralization are more susceptible to Mtb infection, exhibiting uncontrolled bacterial proliferation and disorganized granulomata. In humans, treatment with TNF blocker infliximab caused extra-pulmonary disease in 50% of treated patients and disseminated disease in 10% of patients (175). In fact, TNF-α has

been suggested to play a key role in the organization of the granuloma and, consequently, in the containment of Mtb. In support of this hypothesis are the studies showing that TNF-α blockers are associated with reactivation and dissemination of other diseases which are controlled by the granulomatous response (176).

2.2.2. Th2 cells

In strike contrast with Th1 responses, Th2 cells participate greatly in activating and maintaining humoral responses and are capable of counteracting Th1 functions. Th2 cells differentiate in the presence of IL-4 and are characterized by the upregulation of the transcription factor GATA Binding Protein 3 (GATA3) and by the production of IL-4, IL-5, and IL-13. As Th2 cells are associated with the recruitment of eosinophils, they are mostly relevant in the protection against helminth infections. Nevertheless, the role of Th2 responses in the context of Mtb infection is controversial. In humans, progression to active TB was found to be associated with the presence of a Th2 cytokine signature in the BALF, pleural fluid, sputum and serum (177–179). In mice, the role Th2 cytokines during Mtb infection is not clear. While IL-4 or IL-4Rα deficiency in B6 mice does not impact survival or lung bacterial burdens (180), IL-4 deficiency in BALB/c mice decreases bacterial proliferations and reduces lung pathology (181). In accordance with human data, TB reactivation in BALB/c mice is correlated with increased Th2 responses (182). It has been described that mouse genetic background is important for the Th1/Th2 balance and, consequently, for the outcome of Mtb infection (183). In this regard, B6 mice preferentially induce Th1 responses with high levels of IFN-γ, whereas BALB/c mice favor Th2 responses with elevated IL-10 production (183, 184). Therefore, the impact of Th2 cytokine-deficient mouse models in the control of infection is only observed when used in mice with significant Th2 responses. Accordingly, transgenic B6 mice overexpressing IL-13 exhibit increased Mtb proliferation in the lungs, development of extensive pulmonary inflammation with centrally necrotizing granulomas surrounded by a collagen capsule, and reduced survival (185). Interestingly, these mice also present an elevated induction of arginase-1-expressing macrophages in the lungs, which is consistent with the detrimental role of alternatively activated macrophages during Mtb infection discussed in the previous section.

2.2.3. Th17 cells

In addition to Th1 and Th2, $CD4^+$ T cells may also differentiate into a Th17 phenotype. This $CD4^+$ T cell subset is driven by the expression of IL-6, transforming growth factor beta (TGFβ) and IL-1β, and is sustained by IL-23. Th17 cells are characterized by the expression of the transcription factor Rorc RAR-related orphan receptor gamma (RORγt) and display a secreting cytokine signature composed mostly by

IL-17, IL-21, IL-22, TNF and G-CSF. IL-17 is the signature cytokine produced by Th17 cells and comprises a family of cytokines with six members: IL-17A to IL-17F. Although the role of IL-17B-17E is poorly described, IL-17A (often described only as IL-17) and IL-17F share similar structure and roles in the context of intracellular infections (186). Both IL-17A and IL-17F bind to IL-17RA to mediate the induction of proinflammatory genes coding cytokines (G-CSF, IL-6 and IL-8 (or MIP-2, in mice), CXC chemokines and antimicrobial peptides that together modulate granulopoiesis in addition to promote the recruitment and activation of neutrophils (187). As described above, neutrophil recruitment can have a detrimental impact for the control of TB. In accordance, the role of IL-17 during Mtb infection is conflicting and is believed to depend on the route and dose of infection as well as the virulence of the Mtb bacteria. Studies using IL-17 or IL-17RA deficient mice revealed that IL-17 is dispensable for protective immunity against Mtb in mice infected with a low-dose via the aerosol route of the lab strain H37Rv, as these mice were capable of controlling Mtb growth in these conditions (188). On the other hand, mice with a genetic or antibody-mediated blockade of IL-17 signaling and infected with a ten-fold higher dose of H37Rv through intratracheal injection were not capable of maintaining the long-term control of Mtb (189, 190). Indeed, despite the controlled bacterial proliferation early during infection, these mice presented higher lung bacterial burdens, increased inflammation and reduced survival three months after infection (189, 190). A different study showed a distinct phenotype for the same conditions in which mice deficient for IL-17A production displayed increased lung bacterial burdens and reduced inflammatory infiltrates by the first month of infection (191). Importantly, all these studies showed a reduced early accumulation of neutrophils, macrophages, and lymphocytes. Strikingly, absence of IL-17 signaling during the infection with hyper-virulent Mtb clinical isolate strain HN878 is associated with increased lung bacterial burdens and disorganized granuloma structures, independently on the dose of infection (188). Thus, IL-17 appears to have a more relevant role in the protective immunity against more virulent Mtb strains. Importantly, infection with HN878 revealed that the role for IL-17 in mediating protection against Mtb infection is independent of neutrophil recruitment (188). Instead, IL-17 stimulates the production of the chemokine CXCL13 by IL-17R-expressing stromal cells and DCs in the lungs. CXCL13 expression not only recruits B cells, but it also attracts T cells expressing its receptor, CXCR5, towards tertiary lymphoid structures (TLS) developed in the lungs with the course of infection. The infiltration of T cells in these structures assists their engagement with infected cells, thus improving control of infection (188). The development of TLS and their relevance during Mtb infection will be discussed in section 3.2 of this chapter.

The role of IL-17 in human studies is also inconsistent. Some studies support a protective role for IL-17 since its expression was shown to be associated with the transition from active to latent TB (192,

193). However, other studies showed that a higher IL-17 expression by T cells has been observed in multi-drug resistant strains of Mtb which have important immunopathological consequences to the host (192, 194). Additionally, a reduction in IL-17 expression has been observed in patients receiving treatment for TB (194, 195). One of the possible explanations for these different observations is the type of human sample used. Many studies with human cohorts analyze immune responses from peripheral blood cells. However, it has recently been showed that plasma levels of IL-17 differ significantly from IL-17 production at the site of infection (196). The same study discovered that the infected human lung tissue is highly enriched with a functional T cell population with a tissue-resident memory-like phenotype producing high levels of IL-17 upon restimulation with Mtb peptides that is, in animal models, associated with improved protection against Mtb (196).

Despite being traditionally described to induce neutrophil recruitment to the site of infection, IL-17 has also been described to be key in vaccine-mediated protection by inducing Th1 responses (197–200). Mice vaccinated either through the subcutaneous or mucosal route with the immunodominant antigen ESAT-6, display an IL-17-dependent early accumulation of antigen-specific IFN-γ-producing $CD4^+$ T cells in the lungs following a standard low-dose aerosol infection with Mtb H37Rv, when comparing with naive mice (198). Accordingly, the adoptive transfer of ESAT-6-specific Th17 cells into naive hosts results in a recall response that confers protection to levels similar to that observed for vaccinated mice (199). Similarly to what was observed in HN878-infected mice (188), IL-17 expression in these mice induced the production of CXCL13 that was key for the strategic T cell localization within the TLS. Therefore, IL-17-dependent protective mechanisms are likely to rely on inducing improved and more efficient Th1 responses. Although the role for Th17 cells in mediating the accumulation of protective IFN-γ-producing $CD4^+$ T cells has not yet been described for BCG vaccinated hosts, Th17 cell responses are increased in BCG vaccinated animals and humans (193, 201, 202). Importantly, IFN-γ is described as a negative regulator of Th17 responses (203). Thus, the protective vs pathological role for IL-17 in the context of Mtb infection can potentially rely on a tight balance between Th1 and Th17 responses. As discussed above, several innate immune cell populations also produce IL-17, especially in the mucosa. Indeed, recent studies suggest that this might be one of the factors explaining the improved protection observed for mucosal vaccination when comparing with the traditional parenteral administration (204, 205). Thus, the cellular sources and precise mechanisms whereby IL-17 mediates protection in BCG vaccinated hosts remain to be fully defined.

2.2.4. T regulatory cells (Tregs)

$CD4^+$ T cells also play a part in regulating inflammatory responses. In this regard, T regulatory (Treg) cell population constitute an essential antigen-specific population capable of counterbalance the inflammatory counterparts and are pivotal to sustain immune homeostasis and peripheral tolerance. Tregs express the transcription factor forkhead box P3 (Foxp3) and can quickly expand during the early stages of the infection and persist during chronic Mtb infection (206). In addition to Foxp3, Tregs are often characterized by CD25 expression, although T cells with regulatory properties were also found in the CD25-negative Treg subset (207, 208). Importantly, IL-10 has been shown to act on Tregs to increase their proliferative and suppressive capacity (209). Tregs can act through different mechanisms. The production of immunosuppressive cytokines such as IL-10, TGFβ, and IL-35 (210–212) is widely recognized as an essential mode of action employed by Tregs to downregulate inflammatory processes. In addition to indirect effects, T regs also act directly on APCs, mostly DCs, to weaken their maturation and, consequently, impair the differentiation of pro-inflammatory subsets of T cells. Particularly, Tregs promote the downregulation of the costimulatory molecules CD80/CD86 by DCs (213, 214). These molecules bind to CD28 receptor in the surface of T cells and are essential for antigen presentation and subsequent T cell activation and differentiation. Tregs may also engage via cytotoxic T-lymphocyte antigen 4 (CTLA-4) with CD80/CD86 molecules from DCs to induce their production of immunosuppressive enzymes, mainly indoleamine 2,3-dioxygenase (IDO) (215, 216). IDO acts on effector T cells by inducing the production of pro-apoptotic metabolites that contribute for the suppression of T cell effector functions (215, 216). In addition to these modes of action, Tregs also express granzyme and perforin and can also directly induce cytolysis in a process widely considered the forte of NK and $CD8^+$ cells (215, 217).

As with many other immune players, the beneficial vs pathological effect of Tregs is the context of Mtb infection is debatable. Many studies using human blood samples show higher numbers of Tregs in active TB patients when comparing with latently infected individuals or healthy controls (218, 219). Some of these studies show similar results using the follow-up of TB patients under treatment (220). Despite this, it is still not clear whether high levels of Tregs are a consequence of inflammation or a risk factor for disease. Animal models can be useful to approach this issue. In Mtb-infected NHP, there are evidence that Tregs accumulate in response to inflammation, rather than being the cause of disease (221). On the other hand, depleting CD25 prior to Mtb infection of B6 mice did not impact the outcome of the infection (222). However, the same was not observed in DBA/2 mice, as the outcome of infection improved with the deletion of CD25-expressing cells (223). This difference is likely a consequence of the distinct inherent susceptibility against Mtb infection, and the differential intrinsic production of IL-10 (224).

2.2.5. T follicular helper (Tfh) cells

In addition to conventional Th subsets, a specialized subset of $CD4^+$ T cells, Tfh cells, was more recently identified in secondary lymphoid organs (SLOs). The main function of the Tfh cell subset is to assist in B cell activation and antibody production within the SLO (225). Within SLOs, T cell zones surround B cell aggregates and are constituted by $CD4^+$, $CD8^+$ T cells as well as conventional DCs (226). On the other hand, B cell clusters are grouped around stromal cells, mostly follicular dendritic cells (fDCs), and can contain germinal centers (226, 227). It is within the germinal centers that B cells undergo rapid proliferation and affinity maturation to allow the production of antibodies with greater affinity for their targets (226, 227). Furthermore, it is in the germinal center that B cells differentiate into memory B cells. Tfh cells interact directly with B cells via the co-stimulatory molecule CD40 and produce IL-21 to drive B cell proliferation (225). Hence, Tfh cells have a determinant role in antibody production since their presence is critical for the secretion of antibodies with high affinity.

Tfh cells are characterized by the expression of the transcription factor B cell lymphoma 6 (Bcl6) as well as cell surface markers CXCR5, programmed cell death protein 1 (PD-1) and inducible T cell costimulator (ICOS) and have been shown to be present in the lungs of mice, NHP, and humans infected with Mtb (228). Similar to classical helper T cells, Tfh cells differentiate within the T cell zones of the SLO (225). The production of IL-6 by APCs induces upregulation of CXCR5 and down-regulation of CCR7, facilitating their migration into the B cell zone where they will become mature Tfh cells and assist in antibody production (225). It has been shown that active TB patients present decreased frequencies of circulating Tfh cells and plasma IL-21 levels (229). Other studies have described the beneficial role of the accumulation of a Tfh-like $CD4^+$ T cell population also expressing characteristic surface markers of Th1 subset in the lungs of Mtb infected animals (228). Overall, the role for this subpopulation of Th cells in conferring protection against Mtb infection is an emerging topic and requires further attention.

2.2.6. Defining features of protective $CD4^+$ T cells

The experimental and clinical data discussed above clearly demonstrate that the presence of IFN-γ-producing $CD4^+$ T cells is key for host resistance against Mtb infection. Accordingly, the protection conferred by different vaccination approaches in experimental models is associated with the presence of Mtb-specific cytokine-producing $CD4^+$ T cells (230–234). Consequently, current vaccination strategies aim at boosting the numbers of IFN-γ-producing $CD4^+$ T cells in the lungs. However, recent efficacy trials for new vaccine regimens reveal that promoting the expansion of these cells does not improve protection beyond the protection conferred by BCG (235, 236). In fact, it has been shown that increasing the levels

of IFN-γ-producing CD4+ T cells can even have immunopathological consequences with earlier death of the infected host (237), highlighting a potential pathological role of the T cell response in the context of Mtb infection. Therefore, the presence of strong IFN-γ-producing CD4+ T cell responses does not correlate with host resistance against Mtb infection (238–240). These observations indicate that other features of the CD4+ T cell response must be responsible to mediate protection against Mtb.

Experimental data using animal models revealed that the proper activation of effector CD4+ T cells requires direct contact with Mtb-infected cells within the lung (170). This observation urged the hypothesis that the ability of CD4+ T cells to exit circulation and successfully penetrate the lung is a key feature of protective CD4+ T cells against Mtb. Accordingly, the scientific community has been making increasing efforts to understand what drives the accumulation of CD4+ T cell in the infected lungs, and how this impacts their phenotype and ability to control bacterial proliferation. The inability of CD4+ T cells to penetrate the parenchyma has also been associated with their degree of polarization and functional exhaustion. In both mouse and non-human primate models, lung parenchyma-residing CD4+ T cells are characterized by CXCR3 expression while their vasculature-residing counterparts express CX3CR1 (241, 242). CXCR3-expressing CD4+ T cells also present increased expression of CD69, and of the inhibitory checkpoint molecule PD-1. Although it is likely that increased expressions of CD69 and PD-1 occur due to increased antigen exposure, these CD4+ T cells do not present signals of exhaustion and are a self-renewing population with marked proliferative capacity (243). In contrast, vasculature-associated CD4+ T cells present a terminally differentiated phenotype characterized by increased killer-cell lectin like receptor G1 (KLRG1) expression (243). Adoptive transfer experiments using T-cell depleted recipient mice demonstrated that donor parenchymal PD-1+CD4+ T cells exhibit improved migration into the lung parenchyma and mediate the most efficient control of Mtb infection (243). Importantly, within the lung parenchyma, T cells require additional signaling to exit the perivascular lesion and move towards the nascent granuloma (Figure 3).

This immunoregulatory molecule PD-1 has been previously shown to be expressed by activated T cells upon antigen exposure, to prevent their rapid proliferation and cytokine secretion. Indeed, recent data suggest that PD-1 expression is essential to limit T cell-mediated immunopathology in chronic infections (244). PD-1-defficiency in the murine model of TB results in uncontrolled expansion of CD4+ T cells, leading to critical immunopathological consequences to the host (143, 245). In recent studies using NHP, PD-1 blockade also exacerbated disease progression by delaying the accumulation of Mtb-specific CD4+ T cells in the blood and lungs and elevating pro-inflammatory cytokine production (246). In humans, the association between the presence of PD-1+ CD4+ T cells and control of infection is also an emerging

topic. PD-1 was found to be elevated in tissue-resident $CD4^+$ T cells from human patients but not in areas of persistent immunopathology (247), supporting a protective role for PD-1 in regulating T cell-mediated immunity. Consistently to what was found for the mouse model, $PD\text{-}1^+$ $CD4^+$ T cells from human lungs displayed a characteristic tissue-resident phenotype with a predominant CD69 expression and, in smaller proportions, CD103 expression (247). The protective role for PD-1 during Mtb infection is also supported by the well described development of TB in cancer patients subjected to PD-1 blockade (248–252). These data show that the PD-1 pathway is required to restrain pathological T cell responses and prevent severe TB disease. Intriguingly, PD-1 expression appears to be specifically driven by chronic Mtb antigenic stimulation and has, consequently, been studied as a marker of Mtb latency. Indeed, a study using BCG-vaccinated individuals either uninfected or with latent TB revealed that infected patients harbored memory $CD4^+$ T cells expressing high levels of PD-1 when comparing with the uninfected counterparts (253). Additionally, PD-1 expression was also found to distinguish individuals with clinically resolved TB from patients diagnosed with latent TB that expressed high levels of PD-1 (253).

On the other hand, KLRG1 expression has been shown to be detrimental for the control of Mtb infection. Mice deficient in KLRG1 have increased survival, lower lung bacterial burdens and reduced pathology throughout infection (254). Also, KLRG1 expression in lung $CD4^+$ T cells was found to decrease after anti-Mtb chemotherapy in mice (255). During pre-clinical studies, the development of $KLRG1^-$ $CD4^+$ T cells was associated with increased protection of vaccine candidates (256–258). Accordingly, the progressive loss of BCG vaccine-induced protection was also found to be associated with a gradual increase in KLRG1 expression by lung $CD4^+$ T cells (259). These findings suggest that KLRG1-expressing $CD4^+$ T cells represent a cell population associated with impaired immunity against Mtb infection.

Recent efforts have been directed to understand the factors that lead to the generation of protective $PD\text{-}1^+$ $KLRG1^-$ $CD4^+$ T cells. In the murine model, Mtb-specific $PD\text{-}1^+$ cells express both effector and memory-associated markers (260). For instance, Bcl6 expression supports the generation of protective $PD\text{-}1^+$ $CD4^+$ T cells while ICOS signaling was shown to promote and maintain PD-1 expression by Mtb-specific $CD4^+$ T cells during chronic antigen exposure (260). On the other hand, IL-27, a cytokine produced both in humans and mice during Mtb infection and associated with poor control of infection, was shown to promote the generation of terminally differentiated CD4 T cells associated with the lung vasculature (169).

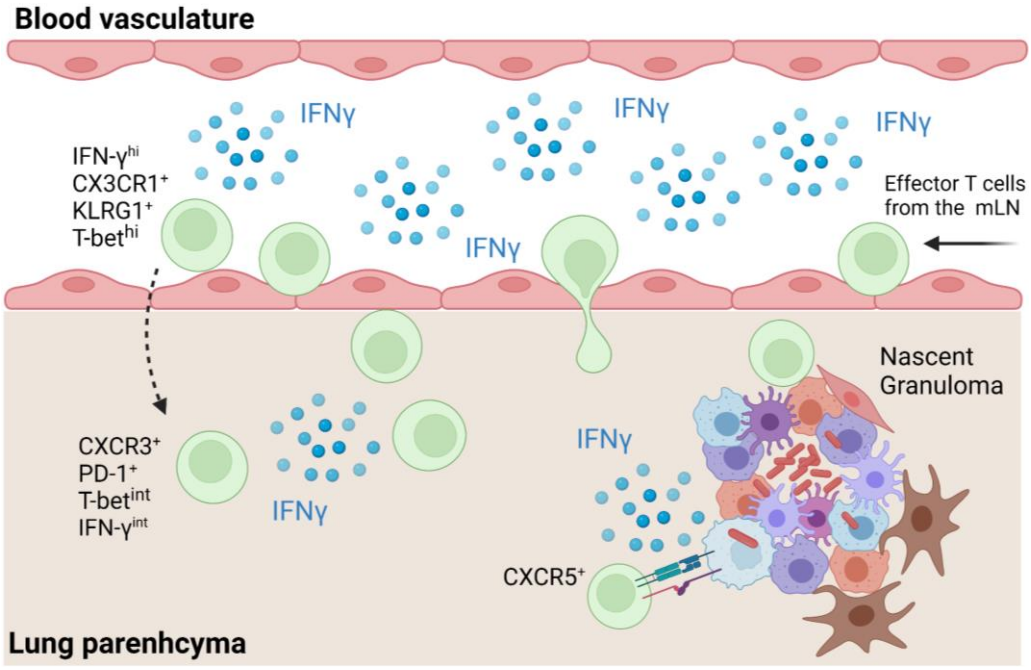

Figure 3- Efficient migration of CD4⁺ T cells into the lung parenchyma is essential for the control of *M. tuberculosis* infection. Antigen-specific effector T cells exit the mLN and recirculate the bloodstream mediated by a chemokine gradient that induces their accumulation in the lungs. Efficient T cell migration from the blood vessels into the lung parenchyma is crucial for their penetration into the innate inflammatory aggregates formed during early stages of the infection (nascent granuloma), where they will intensify antimicrobial effector functions by infected cells. The rate of T cell migration into the lung parenchyma is associated with their CXCR3 expression, and CXCR5 is required for optimal focusing of T cells away from the perivascular region and toward the macrophage-dominated lesion. The combined expression of chemokines by the inflamed tissue and of chemokine receptors by effector T cells is crucial for the optimal control of infection in the lung. Created with BioRender.com.

Interestingly, vasculature-associated KLRG1⁺ CD4⁺ T cells produce more IFN-γ and display higher expression of T-bet than its parenchymal counterpart (169, 241). Further studies using T-bet-deficient and T-bet-haploinsufficient mice showed that T-bet expression is crucial for the development of terminally differentiated CD4⁺ T cells (169, 261). Indeed, T-bet haploinsufficient mice display reduced expression of T-bet and do not harbor terminally differentiated T cells. Crucially, these mice are more resistant to Mtb infection (169, 262). These results highlight the dichotomy of IFN-γ-producing CD4⁺ T cells in the lungs of Mtb infection. In accordance, human data show that active, but not latent, TB disease is characterized by an overly intense IFN-γ-producing CD4⁺ T cell response (263). Therefore, regulation of the CD4⁺ T cell response is critical to improve the outcome of TB disease. The duality between PD-1⁺ and KLRG1⁺ subpopulations show that CD4⁺ T cells can simultaneously protect and harm the host during Mtb infection.

Besides location, a timely response is essential to develop protective immunity against infection. During Mtb infection, there is a delayed priming of the T-cell response. This subject has been mostly addressed using the aerosol mouse model since the initiation of acquired immune responses are difficult to address in humans, due to limitations in sampling and determining time of exposure. In Mtb infection,

T cell priming follows bacterial dissemination to the mLN, held mostly by DCs, and occurs 7-14 days after infection (125, 137, 264, 265). Only by day 21 post-infection the accumulation of antigen-specific $CD4^+$ T cells in the lungs reaches a maximum threshold and bacterial growth is controlled (125, 137, 264, 265). This kinetics contrasts with the one found for T cell activation against other bacterial infections, such as *Listeria monocytogenes,* wherein priming and expression of T cell immunity occurs 3-7 days after infection (266). Importantly, this delay is correlated with susceptibility to infection. Indeed, Mtb appears in the mLN earlier in a relatively resistant mouse strain B6 than in a more susceptible strains such as C3HeB/FeJ mice (267). Additionally, it has also been described that the susceptibility of IL-12p40-deficient mice is associated with a reduced migration of DCs to the mLN due to their downregulation of CCR7. Consequently, these mice also exhibited impaired $CD4^+$ T cell activation in the mLN (268). Treatment of these mice with IL-12p40 homodimers restored DC trafficking to the mLN and $CD4^+$ T cell priming. Particularly, depletion of $CD11c^{hi}$ $CD11b^{hi}$ DCs in mouse models revealed that this subset of DCs is essential for the rapid initiation of the $CD4^+$ T cell responses specific against ESAT-6 antigen (266, 269, 270). Importantly, the delayed accumulation of T cells within the lungs is likely associated with impaired migration from the vasculature into the parenchyma discussed above. In this regard, the work described in Chapter II supports this hypothesis.

Cognate interactions between antigen-specific $CD4^+$ T cells and antigen/MHC complexes expressed by infected macrophages are required to restrict Mtb growth. Therefore, it seems reasonable that the nature of the Mtb antigen recognized by $CD4^+$ T cells also governs the ability of these cells to mediate protection. Numerous Mtb antigens are recognized by $CD4^+$ T cells throughout infection. ESAT-6, Ag85A, and Ag85b, elicit the strongest immune responses being, therefore, the ones most used in vaccine development. Ag85A and Ag85B are enzymes involved in mycobacterial cell wall synthesis and have been demonstrated to be downregulated after three weeks of infection in the mouse model (271). On the other hand, ESAT-6, a secreted virulence factor, is produced and recognized by T cells throughout Mtb infection in mice and humans (260, 272). Interestingly, it has been demonstrated that both in mice and in vaccinated humans, antigen availability limits immunity conferred by Ag85b-specific $CD4^+$ T cells, which maintain memory cell features throughout infection (272). On the other hand, Mtb infection drives ESAT-6-specific $CD4^+$ T cells towards terminal differentiation, restricting effective immunity. These observations could explain why post-exposure vaccines containing ESAT-6 and no other antigens, including Ag85B, were capable of conferring protection in mice (273). Additionally, this observation could also indicate a potential caveat for BCG vaccination. Therefore, infection stage-specific differences in antigen availability should be considered in the design of TB vaccines.

Currently, there is an appreciation for the importance of extending our comprehension of antituberculosis $CD4^+$ T cell effector mechanisms beyond IFN-γ production. Vaccination studies have been demonstrating that $CD4^+$ T cells are pivotal for protective immunity against a primary and secondary encounter with Mtb. Migration studies revealed that $CD4^+$ T cell translocation into the lung parenchyma is essential for their interaction with infected cells and exert protection against infection (169, 170, 228). Hence, a better understanding of the mechanisms contributing for improved $CD4^+$ T cell interaction with infected cells, and the link with terminal T cell differentiation is essential. Integrating these new perceptions will likely provide logical approaches for the development of novel strategies for the development of preventive interventions for TB.

2.2.7. Regulation of the immune response: the special role of IL-10

During infection, the host must respond with sufficient intensity and duration to control the infection. Nevertheless, the expression of immune regulatory mechanisms must be tightly balanced, so that it does not mediate immunopathological consequences to the host. Indeed, as discussed in the previous section, immune regulatory mechanisms, such as the expression of coinhibitory molecules, are essential to prevent immunopathology but may also promote susceptibility to Mtb infection. Therefore, the mechanisms that regulate immunity, particularly $CD4^+$ T cell responses, must be explored beyond current knowledge.

In addition to PD-1 and KLRG1, other coinhibitory molecules are expressed by $CD4^+$ T cells to regulate the stimulation of infected cells during chronic Mtb infection. Accordingly, patients diagnosed with latent TB were shown to present increased expression of PD-1, CTLA-4, and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) by T cells when comparing with healthy controls (274). These patients displayed a reduction in the expression of these markers after TB treatment (274). Accordingly, the expression of PD-1, TIM3, and KLRG-1 has also been shown to change in coordination with alterations in bacterial burdens in mice (255). The expression of specific inhibitory molecules has also been speculated to correlate with the stage of infection. While $CD4^+$ T cells expressing PD-1 are common in patients with latent TB, CTLA-4-expressing $CD4^+$ T cells are found in patients with active TB (275). In the murine model, CTLA-4 blockade reverts the suppression of effector T cell responses by Tregs (276). Despite this, CTLA-4 blockade does not improve protection against Mtb infection in mice (277). The precise mechanisms through which these inhibitory receptors mediate control of infection remain to be clarified. TIM3 expression, for instance, has been shown to play two potentially opposing functions during Mtb infection. On one hand, TIM3 expression by Th1 cells expectedly downregulates

their expansion. However, the binding of TIM3 with galectin-9 (Gal9) from infected macrophages induces their activation and assists in restricting intracellular Mtb growth both in mice and humans (255, 278, 279). Therefore, it is possible that some immunoregulatory molecules have evolved to inhibit the growth of intracellular pathogens directly, thereby negatively signaling Th1 expansion to prevent prolonged inflammation.

The co-expression of TIM3 with PD-1 and lymphocyte-activation gene 3 (LAG3) is associated with the production of the anti-inflammatory cytokine IL-10 (280). Indeed, in what regards immune regulation, IL-10 has been shown to play a key role. Although initially considered a cytokine produced by Th2 cells, it is now established that IL-10 is produced by different T cell subsets and myeloid cell populations. Additionally, most hematopoietic cells express IL-10R, being, therefore, a target for IL-10 regulatory functions. In this regard, IL-10 has been described to suppress antigen presentation as well as to reduce the production of pro-inflammatory cytokines, chemokines, adhesion molecules, and co-stimulatory molecules such as CD80/CD86 by APCs (281, 282). These effects on APCs are corroborated by the observed reduced proliferation and IFN-γ production by IL-10-stimulated $CD4^+$ T cells from latent TB patients (283). Accordingly, neutralization of IL-10 signaling in peripheral blood mononuclear cells (PBMCs) from patients with latent or active TB results in increased proliferation and IFN-γ production (281, 284, 285). These observations suggest a key role of IL-10 in susceptibility to TB, both in human and animal models. For instance, SLC11 A1, a TB susceptibility locus, has been associated with increased IL-10 responses (286). Additionally, active TB patients were shown to have increased levels of IL-10 in their BALF (287, 288), and serum (289) when compared with healthy controls. Importantly, this IL-10-induced susceptibility is tightly linked with decreased $CD4^+$ T cell responses, specifically reduced IFN-γ-producing $CD4^+$ T cells (282, 284, 290, 291).

Animal studies have been useful to demonstrate that the increased levels of IL-10 observed in susceptible hosts is a cause rather than a consequence of increased inflammation. Indeed, mice deficient for Bhlhe40, a master regulator of IL-10 expression, display increased lung bacterial burdens associated with the development of large neutrophil-dominated lesions (292). Similarly, transgenic mice overexpressing IL-10 presented reduced capacity to generate Mtb-specific IFN-γ-producing $CD4^+$ T cells, increased lung bacterial burdens, and pathology (224). This phenotype is also observed in the CBA/J mice, wherein the increased levels of circulating IL-10 correlate with exacerbated pathology and reduced survival during Mtb infection (224). Conversely, blocking IL-10 signaling in chronically infected CBA/J mice, results in stabilized pulmonary bacterial burdens and increased survival (293). Importantly, this improvement in the outcome of infection was associated with enhanced IFN-γ-producing $CD4^+$ T cell

responses. The detrimental impact of IL-10 during Mtb infection also extends to vaccination. Indeed, blocking IL-10 signaling during BCG vaccination has been shown to enhance and sustain Th1 and Th17 responses, resulting in increased protection following aerogenic Mtb infection (294).

Despite previous observations, regulatory mechanisms are essential for the control of Mtb infection without immunopathological consequences for the host. In fact, it has been demonstrated that the combined production of Th1, Th17 and IL-10 cytokines within a granuloma contributes to granuloma sterilization (295). Nevertheless, it is likely that the intensity, localization, and timing of IL-10 production has a decisive role in the outcome of infection. Therefore, deeper insights regarding IL-10 function throughout Mtb infection and in the context of vaccination are crucial.

3. Pathology of *M. tuberculosis* infections

3.1. Development, constitution, and regulation of granulomas

One of the hallmarks of human TB pathogenesis is the formation of pulmonary granulomas. These structures are developed in response to chronic antigen exposure and are likely initiated by infected macrophages, as these cells reside at the center of the structure and are the first cells to engulf Mtb (74). The activation of macrophages and DCs and the consequent recruitment of other innate immune cells such as monocytes, NK cells, neutrophils and γδ T cells leads to the development of inflammatory aggregates often termed innate granuloma. Although these inflammatory aggregates inadvertently contribute for the permanence of infection, they also allow antigen upload by APCs and subsequent priming and differentiation of effector T cells in the mLN. Subsequently, activated T cells undergo clonal expansion and, together with B cells, are driven through a chemokine gradient towards the infection site, surrounding the innate inflammatory aggregates (296). This process concludes the formation of the traditional mature granuloma. The mature granuloma is believed to be an adjusted structure developed to facilitate optimal interaction and activation of infected cells. Despite this, granulomas do not always allow sterilization of the tissue. Indeed, studies using NHP models of TB revealed that, within the same animal, there are sterilized and progressing lesions both in latent and active disease (297). However, the mechanisms underlying lesion sterilization remain elusive.

Later during infection, granulomas may undergo structural changes. In this regard, in some granulomas the lymphocytic cuff can become enveloped by a fibrotic capsule that contributes for the containment of the granuloma, although it may also compromise lung function (298–300). Fibrosis is the result of the deposition of extracellular matrix components, mostly collagen. As described above, fibrosis seems to be triggered by signaling pathways that oppose pro-inflammatory processes and may occur

under a microenvironment rich in Th2 cytokines. Indeed, collagen synthesis is mediated by arginase that competes with iNOS for L-arginine as a substrate, thus impairing NO production (95).

Virulent infections can result in the development of necrotic tissue even in the absence of a robust acquired immune response (301). The development of necrosis is detrimental for the control of infection as Mtb is able to grow extracellularly within necrotic debris (298). In addition, necrosis is commonly associated with the formation of caseous necrotic granulomas that frequently results in cavitation. Caseation of a granuloma is associated with active TB, in which the dissemination of the infection and the transmission of the bacteria through coughing occurs. As described above, during severe active TB disease in humans, granulomas no longer restrain Mtb growth and become enriched with neutrophils (226, 302) and permissive monocytes (303). As short-lived cells, when infection clearance does not occur, neutrophils undergo necrosis and discharge intracellular cytotoxic molecules that induce tissue damage and contribute for the extend of inflammation and consequent disease progression (304). Additionally, Mtb is also capable of inducing necrosis in infected neutrophils in humans (305). Therefore, it is reasonable to assume that neutrophils play a relevant role in the development of central necrosis.

Granuloma assembly and maintenance depend on TNF-α production. Antibody-mediated depletion or through genetic ablation of TNF-α results in increased Mtb loads, the development of less defined granuloma structures and with a significant reduction in survival rates (172, 306, 307). Additionally, TNF-α neutralization of chronically Mtb-infected mice also results in the disintegration of the granulomas and decreased survival. In agreement with these data, patients undergoing TNF-α neutralizing therapies exhibit high rates of extra-pulmonary and disseminated TB disease (175, 308–310). Therefore, TNF-α produced by infected cells and, later on, by T cells is pivotal to maintain cell recruitment and maintain granuloma structure, preventing disease reactivation and dissemination.

Several animal models were developed over time to mimic the different characteristics of human immunopathology. Although no single animal model exhibits precisely the same human TB pathology, they all reflect specific aspects of human disease and may contribute to stratify its complexity. Specifically, rabbits and guinea pigs are known to develop necrosis and cavitation (311). However, neither B6 or BALB/c mice develop this phenotype. Despite this, the powerful collection of mouse genetic approaches can be used to mechanistically dissect the processes leading to the development of caseous granulomas. In this regard, specific gene-disturbed mice were already shown to be capable of developing caseous necrosis (312–316).

Altogether, these observations demonstrate that a tight balance between pro- and anti-inflammatory responses during Mtb infection is essential. It is possible that different granuloma microenvironments

exist and comprise cellular populations with distinctive metabolic and immunological features. For instance, the core of an organized granuloma with proliferating bacteria and composed mostly by macrophages requires pro-inflammatory signals to assist in the induction of bactericidal mechanisms. On the other hand, as described, fibrosis surrounding the periphery of the granuloma requires at a certain degree anti-inflammatory signals that activate tissue remodeling. Therefore, while the granuloma structure can shield-off the bacteria and restrain its expansion, it also contributes for Mtb persistence and represents a continuous threat of disease reactivation and dissemination.

3.2. Tertiary lymphoid structures (TLS), a vital component of the granuloma

Tertiary lymphoid structures (TLS) or tertiary lymphoid follicles (TLF) are ectopic organizations of lymphocytes that are frequently found in non-lymphoid tissues with persistent inflammation resulting from an unresolved infection, autoimmunity, or cancer. The organization of the TLS can range from less complex, composed by loose aggregates of B and T cells, to highly organized structures that resemble SLO in their characteristic organization into B and T cell clusters (317). Despite the similarities with SLO, TLS are not encapsulated. Based on the composition and location of the TLS within an organ, different subtypes have been defined. For instance, in the lungs, nodular inflammatory foci (NIF) were shown to develop, in mice, in response to cytomegalovirus and to combine aggregates of myeloid cells and $CD8^+$ T cells (318). However, inducible bronchus-associated lymphoid tissue (iBALT) is one of the most common TLS developed in response to respiratory infections. iBALTs display a similar structure to SLO but are located near perivascular spaces surrounding large blood vessels (319) (Figure 3). The relevance of iBALT has emerged from studies showing that these structures have an important role in disease progression by shaping antigen-specific responses. Accordingly, in the context of Mtb infection, several lines of evidence show that iBALT plays a beneficial role for the control of disease in mice (228, 320–322), NHP (228) and humans (228, 323, 324).

The dynamics of TLS development generally parallels with the embryonic development of a SLO, with the exception that TLS is initiated in response to inflammatory stimuli. In the case of Mtb infection, the presence of specific Mtb genes involved in cell wall formation have already been described to influence iBALT development in NHP (325). This stimulation by Mtb peptides eventually lead to the activation of epithelial cells and alveolar innate immune cells, primarily DCs and macrophages (Figure 4). Particularly, the relevance of DCs in the initiation of this process has been demonstrated in experiments wherein mice depleted of CD11c-expressing DCs fail to maintain iBALT structures whereas the adoptive transfer of bone

marrow-derived DCs into these mice rescues iBALT development (226, 326). The activation of the cells in the first line of defense takes the lead to the production of cytokines, mostly IL-1β and IL-23, that will be responsible for the recruitment of neutrophils (327) and ILCs (317, 328–330), respectively.

As described above, IL-23 contributes for the differentiation of Th17 cells. Nonetheless, IL-23 has also been shown to activate ILC3s that populate the lung mucosa. In this regard, ILC3s have recently emerged as key orchestrators of TLS (336). Indeed, ILC3s mediate early protection against Mtb infection by shaping the organization of iBALT structures through the early production of their hallmark cytokines, IL-17 and IL-22 (205, 226, 321, 331, 332). Accordingly, the vital role of IL-17 and IL-22 for iBALT formation is demonstrated by the decreased B cell area formation developed in the lungs of mice deficient for IL-17 or IL-22 (322). Nevertheless, IL-23-deficient mice display sustained deficient B cell follicle formation during Mtb infection (322), thus highlighting a predominant role for IL-23 in the maintenance of iBALT structures when comparing with IL-17 and IL-22. Importantly, the same study concluded that the susceptibility of IL-23-defficient mice was also associated with the development of T cells with impaired ability to migrate from blood vessels into the infected tissue. Indeed, the relevance of IL-17 and IL-22 appears to arise from their ability to induce the production of homeostatic chemokines by stromal cells that recruit lymphocytes to further engender TLS development at mucosal tissues (320, 333, 334). Among these chemokines are CCL19, CCL21 and CXCL13. CCL19 and CCL21, the ligands for the receptor CCR7, are mostly recognized for participating in the generation of SLO. Nevertheless, these chemokines have also been shown to mediate early antigen presentation in the lymph nodes of Mtb-infected mice by CCR7-expressing DCs and, consequently, to participate in the generation of Mtb-specific effector $CD4^+$ T cells (334, 335). Accordingly, mice deficient in CCL19 and CCL21 showed delayed accumulation of antigen-specific IFN-γ-producing $CD4^+$ T cells (334). These mice also exhibited strong local inflammatory infiltrates but impaired accumulation of B cell aggregates (320). Additionally, CCR7 expression was found to be expressed at high levels in naive and central memory T cells to allow their recirculation and homing into the lymphoid organs (320).

CXCL13 is also a chemokine found in Mtb infected lungs from mice and humans and its production is highly induced by IL-17 and IL-22 (228, 336). The major producers of CXCL13 during Mtb infection are stromal cells, fDCs and $CD11c^+$ DCs (228, 336). Although CXCL13 is a B cell chemoattractant, it has been also linked with the accumulation of Tfh cells. The production of CXCL13 is negatively regulated by the presence of B cells and was found to be up-regulated in B cell-deficient mice (337). Although mice deficient in CXCL13 do not display impaired accumulation of effector $CD4^+$ T cells in the lungs as in CCL19 and CCL21-defficient mice do, the effector $CD4^+$ T cells that accumulate in the lungs are less

efficient at activating infected myeloid cells. In fact, macrophage activation is skewed towards an alternative phenotype in CXCL13-defficient mice (334). This finding suggests that the absence of CXCL13 is required for a more efficient migration of T cells to the infected lung lesions. Importantly, this impaired accumulation of T cells within the granuloma observed in CXCL13-deficient mice associates with increased lung bacterial burdens throughout infection when comparing with WT mice (334). Mice without CCL19, CCL21 and CXCL13 display increased susceptibility when comparing to single knock-out, suggesting that these chemokines act in cooperation for the development and maintenance of protective immune responses against Mtb (334). Mice deficient in CXCR5, the receptor for CXCL13, were also shown to be more susceptible to Mtb infection (228). Likewise, these mice presented T cells with impaired ability to migrate into the lung parenchyma and distribute within the granuloma, which was associated with deficient macrophage activation and reduced formation of TLS (228). Similar to the data from IL-23-deficient mice, the findings using mice without CXCL13 or CXCR5 expression suggest that the mechanisms responsible for the development and maintenance of TLS are also involved in the spatial distribution of Mtb-specific effector $CD4^+$ T cells in the lungs. Therefore, the signaling molecules produced in the context of iBALT development are likely contributing for correct T cell distribution within the nascent granuloma (338).

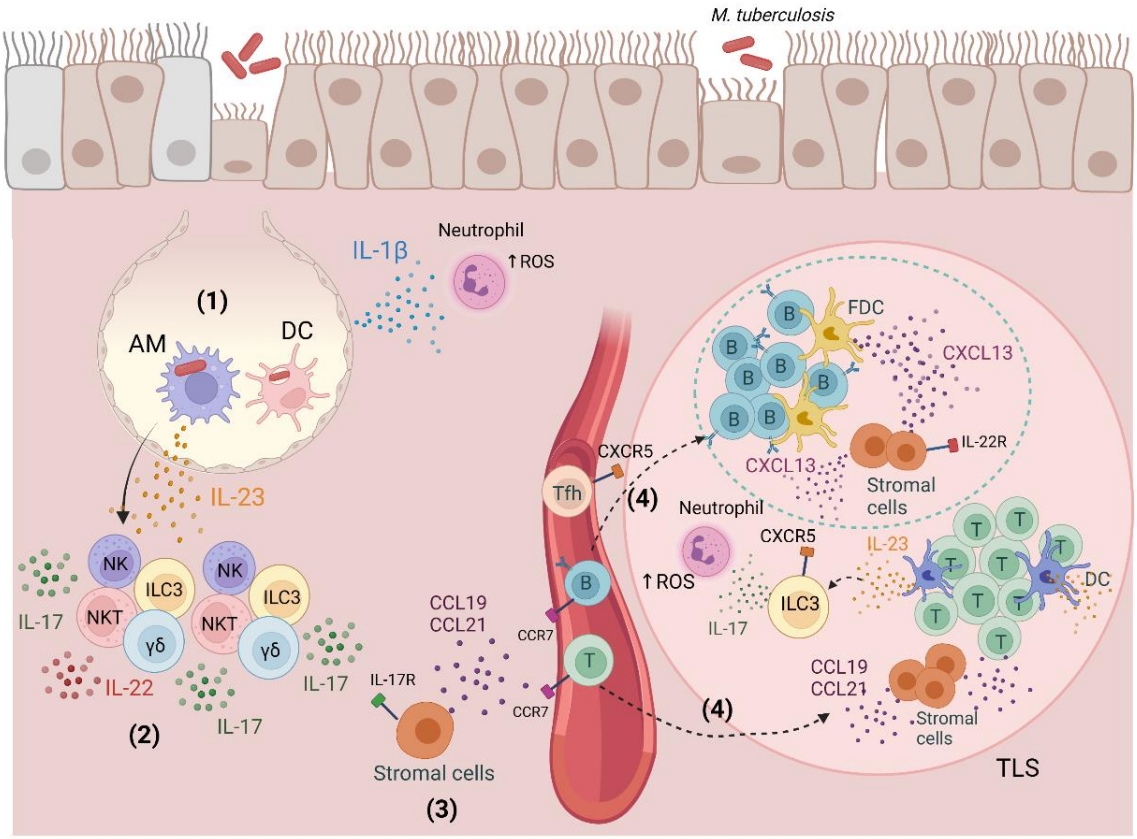

Figure 4- Chronic *M. tuberculosis* infection induces the development of TLS via specific chemokines and chemokine receptors. Upon antigen interaction, host immune cells respond by releasing IL-1β and IL-23 **(1).** Both cytokines will be essential for the recruitment of other innate immune cells and their activation. IL-1β is fundamental for the recruitment of neutrophils into the site of infection, supplying a cellular first line of defense against the pathogen. On the other hand, IL-23 is essential for the production of IL-17 and IL-22 by accumulating and resident innate (NKT, NK, ILC3, γδ T cells) **(2)**. IL-17R-expressing cells such as stromal cells, fDCs and CD11c$^+$ DCs respond to IL-17 by producing chemokines including CXCL19, 21 and 13 **(3).** These chemokines will allow the recruitment of B and T lymphocytes from the bloodstream and their infiltration in the granuloma **(4).** The combined expression of chemokines by the inflammatory cells and of chemokine receptors by effector T cells is important for the development of TLS. Created with BioRender.com.

In addition to the described data, findings from vaccination studies are beginning to support the beneficial role of iBALT development and maintenance during Mtb infection (205, 339). Indeed, NHP vaccinated with attenuated Mtb via aerosol route were shown to mount greater areas of iBALT, following infection, which correlated with a stronger development of memory responses and improved protection (339). Likewise, mice vaccinated with ESAT-6 through the mucosa trigger Th17-mediated CXCL13 induction that assists in strategic positioning of CXCR5$^+$T cells, promoting iBALT formation and conferring protection (205). The development of TLS in the lungs of TB patients has also been described (228, 323, 324, 340). Similar to the data from animal studies, there are evidence that pulmonary lymphoid follicles are scarce or less organized in patients with active disease (340). Accordingly, another study has concluded that 25% of TB patients with active TB show the development of TLS (228). Additionally, there are evidence of higher levels of circulating Mtb-specific B cell accumulation in BCG-vaccinated when

comparing with non-vaccinated individuals (341). Despite this, a clear association between vaccination and the development of TLS in humans is still lacking. Altogether, iBALT formation and maintenance likely contributes for the development of an effective T cell response by modulating its infiltration and interaction with infected cells. Further studies on the subject are needed to understand the potential of iBALT modulation to improve resistance against TB.

Altogether, these data highlight the main immunological processes taking place during Mtb infection. Further exploring the features and mechanisms that constitute the protective immune response against Mtb is fundamental for the discovery of correlates of protection that could be targets for the development of novel therapeutic and preventive strategies to fight TB pandemic.

4. Aims

The fundamental role of cytokine-producing $CD4^+$ T cells in the control of Mtb infection is indisputable. Therefore, novel vaccine regimens that aim to boost these responses have been developed over the last decade. Unfortunately, none of these vaccines has shown improved protection in humans over that conferred by BCG alone. Data from the mouse model revealed that the memory response to mycobacterial antigens has a significant delay in its expression in the lungs following Mtb aerosol exposure. Indeed, BCG-vaccinated mice begin controlling pulmonary Mtb growth only 5 days earlier than unvaccinated mice (198, 201, 342). Therefore, it is critical to define the immune components delaying the expression of $CD4^+$ T cell immunity after vaccination and following infection.

The immunosuppressive cytokine IL-10 emerged as a central negative regulator of the protective T cell-mediated immune response against Mtb. Despite this, the precise mechanisms whereby IL-10 mediates susceptibility to Mtb infection and how these vary during infection remain largely unexplored. Therefore, the central purpose of this Doctoral Thesis is to investigate the key regulatory mechanisms whereby IL-10 shapes the protective pulmonary $CD4^+$ T cell response throughout Mtb infection.

Taking this into consideration, this Doctoral Thesis has the following fundamental aims:

AIM 1: To define the mechanisms whereby IL-10 modulates the pulmonary $CD4^+$ T cell response and impacts disease progression

The detrimental role of IL-10 expression in Mtb infection has been attributed to decreased $CD4^+$ T cell responses. However, the precise mechanisms whereby IL-10 mediates these effects in different stages of infection remain undetermined. One of the difficulties in approaching this topic has been the use of mouse models with constitutive ablation or overexpression of IL-10, limiting the assessment of a temporally distinct effect of IL-10 expression. Therefore, the pMT-10 mouse model used in this doctoral thesis provides an innovative strategy to address the temporal role of IL-10 during Mtb infection.

AIM 2: To assess the effect of IL-10 in the protection conferred by BCG-induced memory $CD4^+$ T cells during Mtb infection.

Previous data indicates that IL-10 modulates the development of memory $CD4^+$ T cell responses during BCG vaccination and impairs protection against Mtb infection. However, the impact of IL-10 expression in the established memory $CD4^+$ T cell response and its consequences in disease progression remain to be elucidated. We use pMT-10 mice to assess the impact of IL-10 in BCG-induced immunity.

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Chapter II

Early IL-10 promotes vasculature-associated CD4$^+$ T cells unable to control *Mycobacterium tuberculosis* infection

This chapter was published in:
Catarina M. Ferreira, Ana Margarida Barbosa, Palmira Barreira-Silva, Ricardo Silvestre, Cristina Cunha, Agostinho Carvalho, Fernando Rodrigues, Margarida Correia-Neves, António G. Castro, and Egídio Torrado. Early IL-10 promotes vasculature-associated CD4$^+$ T cells unable to control *Mycobacterium tuberculosis* infection. *JCI Insight* **2021** 6(21).

CMF contributed to all figures of this work.

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RESEARCH ARTICLE

Early IL-10 promotes vasculature-associated CD4+ T cells unable to control *Mycobacterium tuberculosis* infection

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Cytokine-producing CD4+ T cells play a crucial role in the control of *Mycobacterium tuberculosis* infection; however, there is a delayed appearance of effector T cells in the lungs following aerosol infection. The immunomodulatory cytokine IL-10 antagonizes control of *M. tuberculosis* infection through mechanisms associated with reduced CD4+ T cell responses. Here, we show that IL-10 overexpression only before the onset of the T cell response impaired control of *M. tuberculosis* growth; during chronic infection, IL-10 overexpression reduced the CD4+ T cell response without affecting the outcome of infection. IL-10 overexpression early during infection did not, we found, significantly impair the kinetics of CD4+ T cell priming and effector differentiation. However, CD4+ T cells primed and differentiated in an IL-10–enriched environment displayed reduced expression of CXCR3 and, because they did not migrate into the lung parenchyma, their ability to control infection was limited. Importantly, these CD4+ T cells maintained their vasculature phenotype and were unable to control infection, even after adoptive transfer into low IL-10 settings. Together our data support a model wherein, during *M. tuberculosis* infection, IL-10 acts intrinsically on T cells, impairing their parenchymal migratory capacity and ability to engage with infected phagocytic cells, thereby impeding control of infection.

Introduction

Tuberculosis (TB) remains the worldwide leading cause of death from a single infectious agent. While active intervention is lowering the global incidence of this disease, new tools are required to reach the WHO's ambitious goal of ending TB epidemics by 2035. These tools include improved diagnostics of clinical and subclinical disease and more effective drugs and vaccines (1). Specifically, a more effective vaccination regimen would make a crucial impact to interrupt TB transmission and control TB epidemics (1). Indeed, the current TB vaccine *Mycobacterium bovis* bacille Calmette-Guérin (BCG), while effective in preventing disseminated forms of pediatric TB (2), is not efficient against adult pulmonary disease (3, 4). Therefore, a novel vaccine regimen to protect against pulmonary TB is among the highest global health priorities.

The rational design of more effective vaccines requires a clear understanding of the immunological mechanisms of protection against pulmonary TB. In this regard, a substantial number of data support IFN-γ–producing CD4+ T cells (Th1 cells) as appropriate targets for novel vaccines because these cells are critical to TB control (5–9). Therefore, over the last decade significant efforts have gone into developing new vaccine regimens that elicit strong IFN-γ–producing CD4+ T cell responses or that boost BCG-induced IFN-γ–producing CD4+ T cells. However, the IFN-γ response is not a reliable correlate of protection against TB in humans (10–12). Accordingly, in a recent efficacy trial of modified vaccinia virus Ankara expressing Ag85A from *M. tuberculosis*, the Th1-boosting TB vaccine candidate failed to improve protection in BCG-vaccinated infants (13). While novel concepts for TB vaccination are emerging (14), these data suggest that the rational design of novel TB vaccines relying on cytokine-producing CD4+ T cells requires a better understanding of the crucial components of an effective CD4+ T cell response against *M. tuberculosis* infection.

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: March 29, 2021
Accepted: September 22, 2021
Published: November 8, 2021

Reference information: *JCI Insight*. 2021;6(21):e150060. https://doi.org/10.1172/jci.insight.150060.

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Recent data show that different populations of CD4+ T cells develop in the lungs following *M. tuberculosis* infection (15). CD4+ T cells expressing programmed cell death 1 protein (PD-1) are a self-renewing population that produce low levels of IFN-γ whereas CD4+ T cells expressing the killer cell lectin-like receptor G1 (KLRG1) are short-lived but produce high levels of IFN-γ (15). Crucially, adoptive transfer experiments have unraveled a critical role of PD-1–expressing CD4+ T cells, but not KLRG1-expressing CD4+ T cells, in the control of *M. tuberculosis* growth (16). Moreover, while PD-1 cells express CXCR3 and locate within the lung parenchyma, KLRG1-expressing cells coexpress CX3CR1 and locate in the lung vasculature (16–18). Indeed, KLRG1-expressing cells cuff around blood vessels and do not engage with infected macrophages in the parenchyma, which is critical for the protective function of CD4+ T cells (19). Taken together, these data show that the parenchymal migratory capacity and the ability to engage with infected phagocytes within the lung lesions are critical features of protective T cells. Therefore, it is critical to identify the factors impeding the rapid migration of CD4+ T cells into the lung parenchyma. This will lead to the rational design of vaccines that generate T cells capable of entering the lung lesion and thereby mediate long-lasting protection against TB.

IL-10 has been implicated in increased susceptibility to TB, both in humans and in animal models, an event highly associated with decreased CD4+ T cell responses (20–26). In humans, IL-10 is elevated in the pleural fluid (27), bronchoalveolar lavage (28), sputum (29), and serum (30, 31) of patients with active TB compared with healthy controls. Mechanistically, IL-10 impairs the proliferation of IFN-γ–producing T cells stimulated with *M. tuberculosis* (22, 23). These observations are supported by mouse data wherein the absence of IL-10 in the genetically resistant strains C57BL/6 and BALB/c enhanced the influx of CD4+ T cells into the lungs, resulting in elevated production of T cell–recruiting chemokines and protective cytokines, including IFN-γ, TNF, and GM-CSF (32). Furthermore, the genetically susceptible CBA/J mouse line, and transgenic lines overexpressing IL-10, display enhanced bacterial burdens associated with reduced Th1 responses and impaired macrophage bactericidal functions (24, 25, 33). These data taken together demonstrate that IL-10 has an antagonistic role in the control of *M. tuberculosis* infection; however, the immunological consequences of IL-10 production during TB are currently unknown. Crucially, recent data show that IL-10 blockade during BCG vaccination and following *M. tuberculosis* infection enhances Ag-specific responses and provides significantly greater protection against aerogenic *M. tuberculosis* challenge (34). Therefore, in this work we used a novel mouse model of controlled IL-10 overexpression recently described (pMT-10) (35) to define the temporal role and the mechanisms whereby this cytokine modulates protective immunity to TB. Using this model, IL-10 overexpression was induced in pMT-10 mice by feeding the mice a 2% sucrose solution with 50 mM of zinc sulfate (35).

Our data show that IL-10 overexpression impairs the protective response, prompting uncontrolled bacterial proliferation and severe immunopathological consequences to the host, during the early stages of *M. tuberculosis* infection but not during chronic infection. During the early stages of infection, IL-10 overexpression altered neither populations of antigen-presenting cells (APCs) in the mediastinal lymph nodes (MLNs) nor the kinetics of CD4+ T cell activation. Instead, CD4+ T cells primed in the IL-10–enriched environment were recruited to the lungs but accumulated in the lung vasculature and did not translocate into the parenchyma. This migratory deficit prevented antigen sensing, which limited proliferation and effector cytokine production. Crucially, in adoptive transfer from the lungs of mice overexpressing IL-10 into RAG$^{-/-}$ mice, effector CD4+ T cells primed in the IL-10–enriched environment maintained their vasculature phenotype and were unable to restrain *M. tuberculosis* infection. Together our data support a model wherein IL-10 compromises the protective function of CD4+ T cells by promoting the differentiation of a vasculature-associated phenotype with reduced ability to translocate into the lung parenchyma and infiltrate the infected lesion.

Results

Early but not late IL-10 production impairs the control of airborne M. tuberculosis infection. The progression and severity of TB has been associated with increased levels of IL-10 in both humans (27, 28, 30, 31, 36) and mice (24, 25, 33). Despite this, the immunological consequences of IL-10 overexpression following aerogenic *M. tuberculosis* infection have not been fully defined. We used the recently described pMT-10 transgenic mouse line (35) wherein IL-10 overexpression can be induced at different stages of infection by feeding the mice a 2% sucrose solution with 50 mM zinc sulfate. Inducing IL-10 overexpression allowed us to define the temporal role and mechanisms by which IL-10 antagonizes the protective immune response

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and control of *M. tuberculosis* infection. After aerogenically infecting pMT-10 mice with *M. tuberculosis*, in one group we induced IL-10 overexpression starting at day 5 after infection to determine the early-phase impact of this cytokine in the ability of mice to control *M. tuberculosis* bacterial burdens. To determine the cytokine's chronic-phase impact, in another group we induced IL-10 overexpression starting at day 30 after infection. B6 mice used as controls were also fed with the 2% sucrose solution with 50 mM zinc sulfate used to induce IL-10 overexpression in pMT-10 mice.

We began by testing the impact of IL-10 overexpression during the early stages of infection. After aerosol infection, we found similar bacterial burdens in both groups of mice until day 27 after infection (Figure 1A). While B6 mice controlled *M. tuberculosis* growth from this point onward, bacterial growth continued to increase in pMT-10 mice (Figure 1A). Consequently, pMT-10 mice displayed significantly lower survival compared with B6 mice (Figure 1B). Gross histological examination revealed that, shortly before they succumbed to infection, there were larger lesions in the lungs of pMT-10 mice than in B6 mice (Figure 1C). As expected, the increased susceptibility of pMT-10 mice was associated with increased IL-10 levels in the lungs (Figure 1D), and the blockade of IL-10R activity reverted the susceptible phenotype of pMT-10 mice that now displayed similar bacterial burdens (Figure 1E) and lesion sizes compared with B6 mice (Figure 1F).

We next sought to determine the impact of IL-10 overexpression during the chronic phase of *M. tuberculosis* infection. To do this, we quantified bacterial loads after inducing IL-10 overexpression from day 30 to day 60 or 90 after infection. Compared with B6 controls, pMT-10 mice showed no marked differences in lung bacterial loads (Figure 1G) or lung pathology (Figure 1H).

Taken together, these data showed that IL-10 overexpression during the early stages of the *M. tuberculosis* infection promoted progression of infection and resulted in the hosts' rapid death. On the other hand, IL-10 overexpression induced during the chronic phase of infection significantly altered neither progression nor control of *M. tuberculosis*.

Early IL-10 production delays the accumulation of IFN-γ–producing CD4+ T cells in the lungs. The above data warranted further detailed analysis of IL-10's role during the early stages of *M. tuberculosis* infection. As IL-10–mediated susceptibility to *M. tuberculosis* infection had been associated with reduced Th1 responses in both humans and mice (22, 23, 26, 29, 32, 34), we first asked if *M. tuberculosis*–infected pMT-10 mice displayed impaired CD4+ T cell responses to *M. tuberculosis* infection. To do this, we infected mice and induced IL-10 overexpression starting at day 5 after infection (as above) and analyzed the kinetics of the CD4+ T cell response in the lungs. Compared with B6 mice, pMT-10 mice showed a delayed accumulation of activated CD4+ T cells expressing the activation marker CD44 in their lungs (Figure 2A). To further explore the impact of IL-10 environment in the Ag-specific IFN-γ response, we determined the frequency and number of *M. tuberculosis*–specific CD4+ T cells and their ability to produce IFN-γ. To do this, we restimulated lung single-cell suspensions with the early-secreted antigenic target-6 $(ESAT\text{-}6)_{1–20}$ epitope and determined IFN-γ producers by intracellular cytokine staining (18). In accordance with the above data, we found a delayed kinetics of *M. tuberculosis*–specific IFN-γ–producing CD4+ T cell accumulation in the lungs of pMT-10 mice compared with B6 mice (Figure 2B). Interestingly however, despite the high levels of IL-10, IFN-γ–producing CD4+ T cells increased rapidly in the lungs of pMT-10 mice after day 30 after infection. The kinetics of IFN-γ protein in the lungs of infected mice followed a similar pattern to that of the Ag-specific response determined by flow cytometry (Figure 2C). These data showed that IL-10 production early during infection would delay the accumulation of IFN-γ–producing CD4+ T cells required to control *M. tuberculosis* infection.

IL-10 impairs the priming and effector differentiation of recently activated CD4+ T cells. Reduced frequencies of Ag-specific CD4+ T cells in the lungs can result from defective priming of Ag-specific cells in the lung-draining lymph nodes or defective recruitment to the lungs. Taking into consideration that (a) *M. tuberculosis* dissemination to the MLNs is concomitant with CD4+ T cell priming (37, 38) and (b) IL-10 impairs dendritic cell trafficking to the MLNs (39), we first determined the impact of IL-10 in the transport of *M. tuberculosis* bacteria to the MLNs. To do this, we quantified the bacterial load in the MLNs at a time when CD4+ T cells would first encounter Ag (37). We found similar bacterial loads in B6 and pMT-10 MLNs at days 12 and 16 after infection, showing that IL-10 did not impair the transport of the bacteria from the lung to the MLN (Figure 3A). To determine if IL-10 expression altered the MLNs' inflammatory context, we aerogenically infected B6 and pMT-10 mice with *M. tuberculosis*–mCherry and characterized *M. tuberculosis*–infected myeloid populations at day 14 after infection. For gating strategy, see Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.150060DS1.

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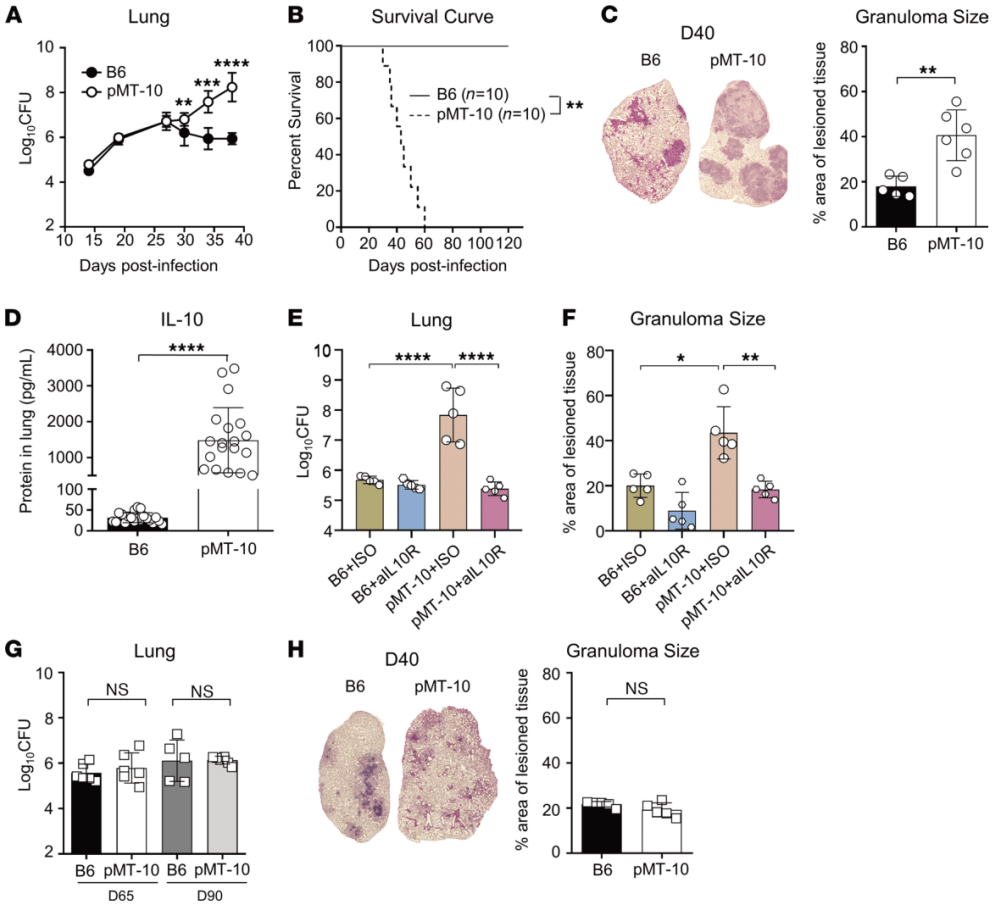

Figure 1. Early, but not late, IL-10 overexpression affects the outcome of aerogenic *M. tuberculosis* infection. B6 and pMT-10 mice were infected with *M. tuberculosis* H37Rv via aerosol route and IL-10 overexpression induced after day 5 after infection (**A–F**). (**A**) Lung bacterial burdens and (**B**) survival of B6 and pMT-10 mice following *M. tuberculosis* infection. (**C**) Representative hematoxylin and eosin–stained lung sections and percentage of infiltrated area in the lungs at day 40 after infection. Individual data points represent individual animals. Lung bacterial burdens and survival and histology data are representative of 3 independent experiments with 4–5 mice per group. (**D**) IL-10 concentration in lung supernatants at day 30 after infection, determined by ELISA. Data represent a composite of 5 independent experiments with 4–5 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{***}P < 0.0001$ using Student's *t* test. (**E**) Lung bacterial burden and (**F**) percentage of lung infiltrated area at day 40 after infection in B6 and pMT-10 mice that were injected weekly with an anti–IL-10R or control Ab starting 1 day before infection. (**E** and **F**) Data are representative of at least 3 independent experiments with 5 mice per group. $^{**}P < 0.01$ by 1-way ANOVA followed by Tukey's test. B6 and pMT-10 mice were infected with *M. tuberculosis* H37Rv via aerosol route and IL-10 overexpression induced at day 30 after infection (**G** and **H**). (**G**) Lung bacterial burden of B6 and pMT-10 mice at days 65 and 90 after infection. (**H**) Representative hematoxylin and eosin–stained lung sections and percentage of infiltrated area in lungs at 65 days after infection. Individual data points represent individual animals. Data represent 3 independent experiments with 4–5 mice per group. Data area shown as the mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{***}P < 0.0001$ by Student's *t* test.

We found that the MLNs from B6 and pMT-10 mice presented similar frequencies of infected cells (Figure 3B). Furthermore, the two strains' profiles of infected cell populations were similar (Figure 3C), the majority of infected cells being $CD11c^{-}CD11b^{hi}$ and $CD11c^{-}CD11b^{lo}$. We additionally analyzed the expression of MHC-II and CD86 and found that, in *M. tuberculosis*–infected cells, IL-10 overexpression did not affect the expression of CD86 (Figure 3D) but it decreased the expression of MHC-II (Figure 3E). These data showed that IL-10 overexpression would not alter the profile of *M. tuberculosis*–infected cells within the MLNs of pMT-10 mice but it impaired MHC-II expression as previously described (40).

We next investigated if these effects of IL-10 had an impact in $CD4^{+}$ T cell priming. To do this, we adoptively transferred CFSE-labeled P25 T cell receptor–transgenic cells (P25Tg, specific for Ag85b presented in the context of $I\text{-}A^{b}$) into B6 or pMT-10 mice at different times after aerogenic *M. tuberculosis* infection and, 24 hours later, determined their activation status (CD69 and CD62L expression) in the MLN (37).

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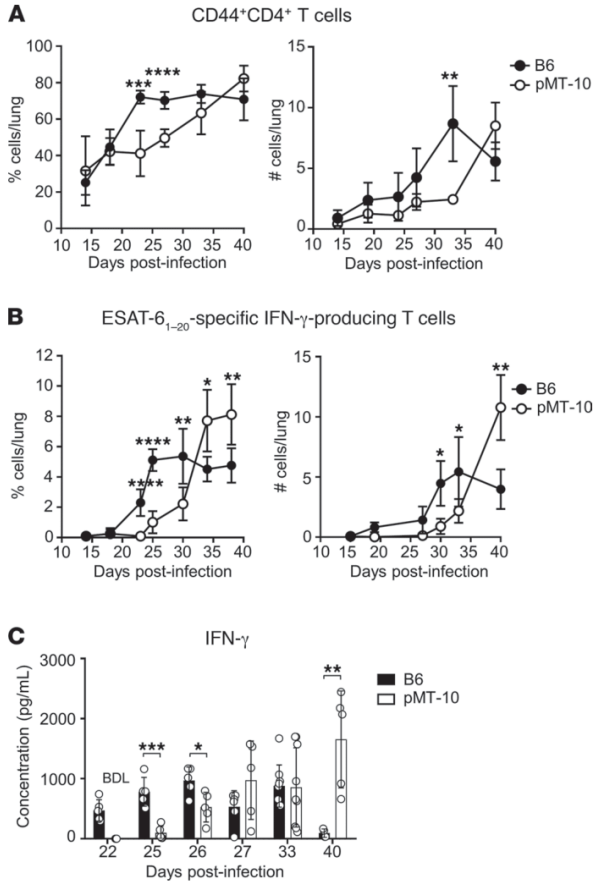

Figure 2. IL-10 overexpression delays the onset of the protective immune CD4+ T cell response. B6 and pMT-10 mice were infected with *M. tuberculosis* H37Rv via aerosol route and IL-10 overexpression induced after day 5 after infection. B6 mice maintained in the same conditions were used as controls. (**A**) Frequency and number of CD4+ T cells in the lungs of mice throughout infection. (**B**) Frequency and number of IFN-γ–producing CD4+ T cells after in vitro restimulation with the immunodominant ESAT-6$_{1-20}$ peptide. (**C**) IFN-γ protein in lung supernatants determined by ELISA. Data represent at least 3 independent experiments with 5 mice per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ***$P < 0.0001$ by Student's *t* test. NS: not significant.

We detected CD69 expression on similar proportions of cells transferred into B6 and pMT-10 mice, up to day 16 after infection, when there was a reduced expression of CD69 evident in P25Tg cells transferred into pMT-10 mice (Figure 3F). We also measured the expression of CD62L in transferred cells and found that, in B6 mice, transferred P25Tg cells lost the expression of CD62L faster than in pMT-10 mice (Figure 3G). This suggested a delay in differentiation of newly activated CD4+ T cells due to the IL-10 environment.

To clarify this issue, we restimulated MLN cells from B6 and pMT-10 mice with ESAT-6$_{1-20}$ to measure IFN-γ production by Ag-specific CD4+ T cells. We found that, at day 14 after infection, there was a reduced production of IFN-γ by MLN cells in pMT-10 mice compared with B6 mice (Figure 3H). Nevertheless, at day 16 after infection, the levels of IFN-γ production were similar in B6 and pMT-10 (Figure 3H).

Together, these data suggested that IL-10 would not significantly impair the kinetics of CD4+ T cell priming although it would delay the differentiation of newly activated CD4+ T cells. Nevertheless, it was unlikely that the delay differentiation before day 16 after infection accounted for the delayed accumulation of IFN-γ–producing CD4+ T cells in the lungs (Figure 2).

Recent data suggested that IL-10 may inhibit the expression of chemokines that guide Th1 cells to *M. tuberculosis* infection sites (32). Therefore, we next asked if IL-10 downregulated the production of chemokines that guide newly differentiated cells to the infected lungs. We quantified CXCL9, CXCL10,

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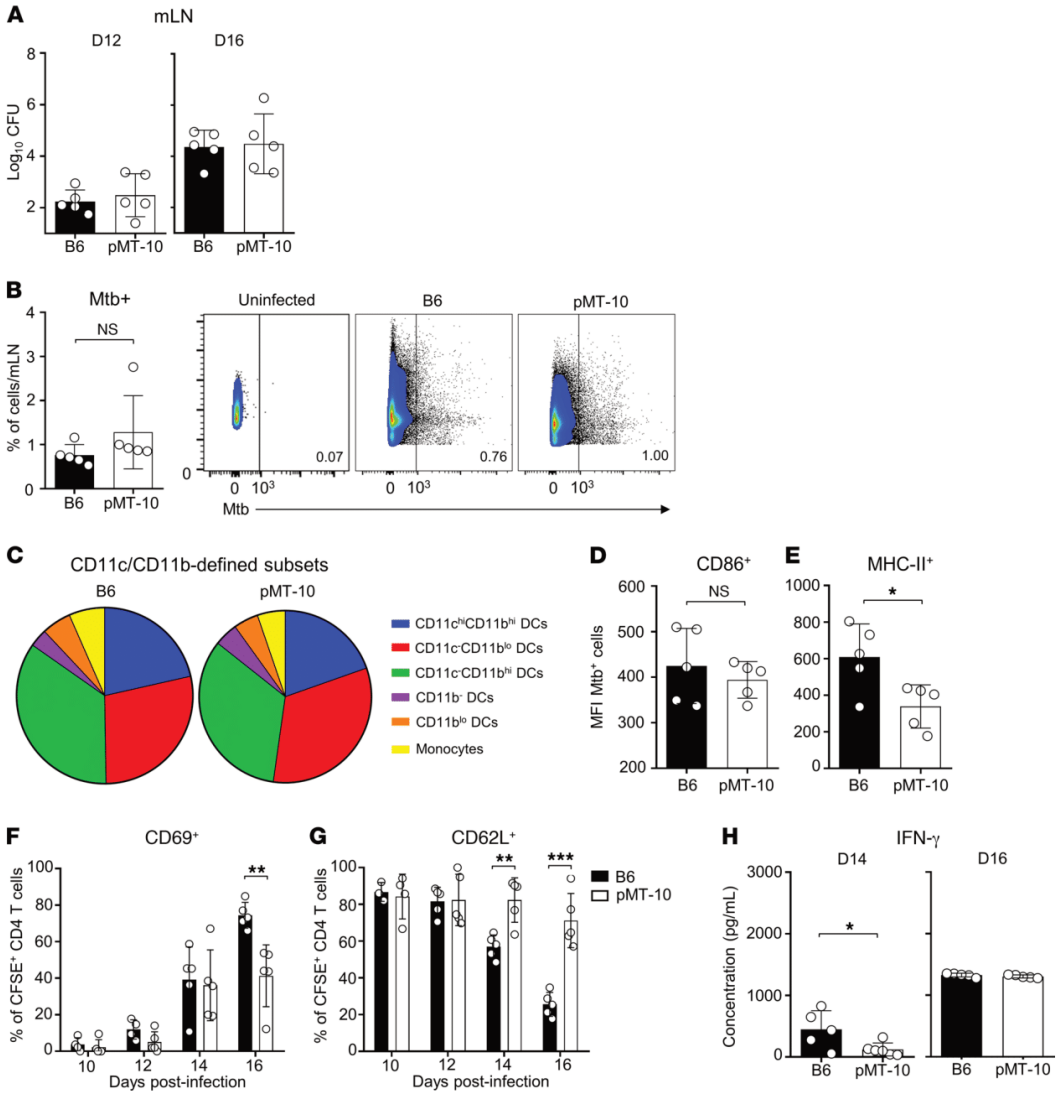

Figure 3. Early IL-10 overexpression does not significantly impair CD4+ T cell priming and differentiation in MLN. B6 and pMT-10 mice were infected via aerosol route with *M. tuberculosis*–mCherry and IL-10 overexpression induced after day 5 after infection. B6 mice maintained in the same conditions were used as controls. (**A**) MLN bacterial burdens at days 12 and 16 after infection. (**B**) Representative FACS plots and frequency of *M. tuberculosis*–mCherry–infected myeloid cells in the MLNs of B6 and pMT-10 mice at day 14 after infection. (**C**) Representative distribution of *M. tuberculosis*–mCherry-infected myeloid cell subsets in the MLNs of B6 and pMT-10 mice at day 14 after infection. (**D**) Mean fluorescence intensity of CD86 and (**E**) MHC-II in *M. tuberculosis*–mCherry-infected myeloid cells from the MLNs of B6 and pMT-10 mice at day 14 after infection. (**F**) Frequency of CFSE-labeled CD4+ T cells expressing CD69 and (**G**) CD62L at different points following aerosol infection with *M. tuberculosis*. (**H**) IFN-γ quantification in supernatants of ESAT-6_{1-20}-stimulated single-cell suspensions prepared from lung-draining lymph nodes of B6 and pMT-10 mice infected for 14 or 16 days. Data represent 2 independent experiments with 4–6 mice per group. Data are shown as the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ***$P < 0.0001$ using Student's *t* test.

and CXCL11 in the lungs of infected mice and found similar protein and expression levels of these chemokines in B6 and pMT-10 mice (Figure 4, A–C). Previous data showed that, in the absence of IL-10, there was an upregulation of CXCL10 production in the lungs of *M. tuberculosis*–infected mice (32). However, the high levels of IL-10 in our model did not impair chemokine production in the lungs following *M. tuberculosis* infection. This response was likely a consequence of the elevated bacterial loads seen in our model. Indeed, we saw enhanced CXCL9 responses in pMT-10 mice after day 25 of infection, the time at which these mice begin displaying higher *M. tuberculosis* loads than B6 mice. As

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such, the data showed that the delayed accumulation of IFN-γ–producing CD4+ T cells was not caused by altered chemokine expression in the lungs.

We then asked if Ag-specific CD4+ T cells from B6 and pMT-10 mice would express similar levels of CXCR3, the chemokine receptor for CXCL9, CXCL10, and CXCL11. Compared with B6 mice, we found in pMT-10 mice that ESAT-$6_{1–20}$–specific CD4+ T cells from the lungs displayed reduced expression of CXCR3 at days 24 and 31 after infection (Figure 4D). Together, these data showed that IL-10 would not impair the production of chemokines that guided CD4+ T cells to the *M. tuberculosis*–infected lungs but it would inhibit the expression of chemokine receptors essential for CD4+ T cells to enter the lung parenchyma and induce control of infection (16, 41).

IL-10 impairs the migration of CD4+ T cells into the lung parenchyma. Our results showed that *M. tuberculosis*–specific CD4+ T cells present in the lungs of pMT-10 mice expressed reduced levels of CXCR3. We therefore hypothesized that IL-10 would antagonize control of *M. tuberculosis* infection by impairing the translocation of CD4+ T cells into the lung parenchyma, where they could interact with infected phagocytes to induce control of infection. In accordance with this hypothesis, the histological examination of lung sections from *M. tuberculosis*–infected mice 30 days after infection revealed the formation of perivascular cuffs in pMT-10 mice whereas in B6 mice lymphocytes localized within the granuloma (Figure 5A). Therefore, we performed intravital flow cytometry to determine the location at days 23, 27, and 33 after infection of CD4+ T cells in the lungs of B6 and pMT-10 mice. To do this, we intravenously injected mice with a fluorochrome-labeled CD45.2 antibody, and mice were culled 3 minutes after injection. Lungs were then collected and processed for flow cytometry analysis as previously described (16). Our results showed that pMT-10 mice presented increased frequencies of intravascular CD4+ T cells at all time points analyzed (Figure 5B). They also presented intravascular CD4+ T cells capable of producing IFN-γ in response to ESAT-$6_{1–20}$ at day 33 after infection (Figure 5C).

The reduced migration of CD4+ T cells to the lung parenchyma of pMT-10 mice was associated with reduced frequencies of CD4+ T cells expressing the early activation marker CD69 at day 23 after infection (Figure 5D). It was also associated with reduced proliferation of CD4+ T cells, as determined by the expression of Ki-67, until day 30 after infection (Figure 5E). Nevertheless, from day 30 after infection onward, pMT-10 mice displayed an increase in the frequency and number of CD4+ T cells expressing CD69 (Figure 5D) and Ki-67 (Figure 5E). This increase was associated with the increased Ag-specific IFN-γ response described above (Figure 2C). Despite this increase, bacterial growth was not curbed by the antigen-specific IFN-γ response. Therefore, we questioned the ability of effector CD4+ T cells generated in a high IL-10 environment to control *M. tuberculosis* infection. To measure this ability, we purified CD4+ T cells from the lungs of B6 or pMT-10 mice at day 30 after infection and adoptively transferred them intravenously into recipient $RAG^{-/-}$ mice infected for 15 days. We then analyzed the accumulation of these cells in the vasculature and their ability to control infection (Figure 6A).

At day 30 after infection, we confirmed that $RAG^{-/-}$:B6 and $RAG^{-/-}$:pMT-10 mice presented similar frequencies of total CD4+ T cells (Figure 6B) and total *M. tuberculosis*–specific IFN-γ–producing CD4+ T cells (Figure 6C) in the lungs. Interestingly, however, we found increased frequencies of intravascular IFN-γ–producing CD4+ T cells in the lungs of recipient mice populated by pMT-10 CD4+ T cells (Figure 6D). These data showed the intrinsic impact of IL-10 in CD4+ T cells' ability to locate within the lungs, independent of the lung microenvironment. Importantly, we found that this phenotype was associated with increased lung bacterial burdens (Figure 6E), highlighting the association between CD4+ T cells' defective spatial distribution in the lungs and susceptibility to infection.

Overall, our data supported a model wherein IL-10 would have a detrimental impact in the development of the acquired immune response. This effect would be mediated by the differentiation of CD4+ T cells that display a vasculature phenotype unable to interact with infected phagocytes to induce control of *M. tuberculosis* growth.

Discussion

Although IL-10 has been associated with increased susceptibility to *M. tuberculosis* infection in humans (27, 28, 30, 31, 36) and in mice (24, 25, 39), the immunological mechanisms underlying this effect are not completely understood. According to the data we obtained in this study, IL-10 overexpression during the early stages of the *M. tuberculosis* infection promotes uncontrolled bacterial proliferation in the lungs and has severe immunopathological consequences as well as reduced survival of the host.

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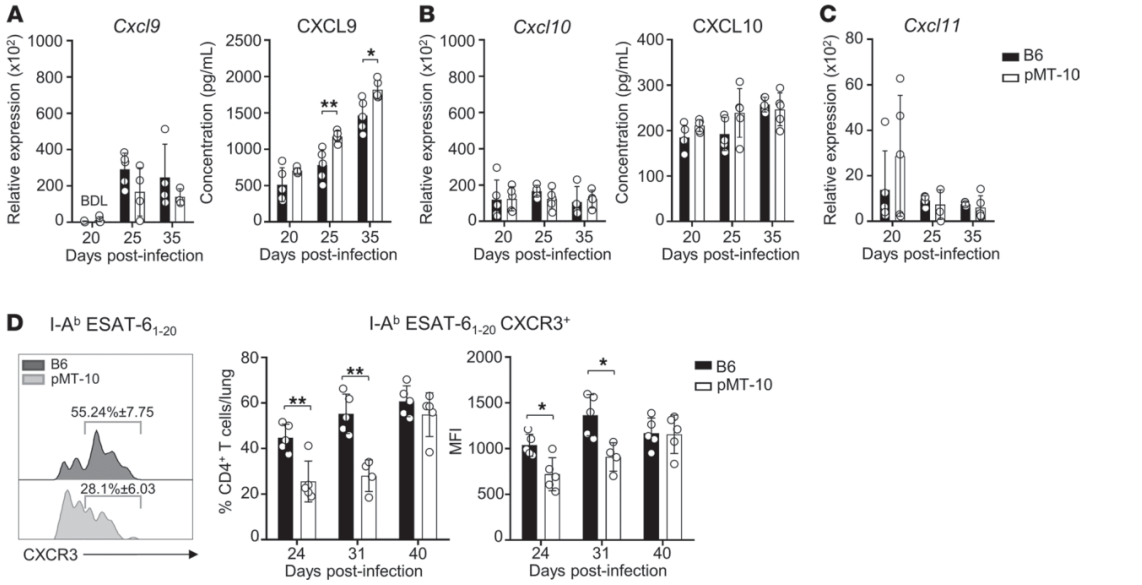

Figure 4. IL-10 overexpression does not impair chemokine production in *M. tuberculosis*–infected lungs. B6 and pMT-10 mice were infected with *M. tuberculosis* H37Rv via aerosol route and IL-10 overexpression induced after day 5 after infection. (**A**) Relative expression and protein levels of CXCL9, (**B**) CXCL10, and (**C**) CXCL11 in the lungs of infected mice. Protein levels of CXCL11 were below detection limit at every time point assessed. (**D**) Frequency of CXCR3-expressing I-Aᵇ ESAT-6_{1-20}–specific CD4⁺ T cells in the lungs of infected mice over the course of *M. tuberculosis* infection. Data represent 2 independent experiments with 4–5 mice per group. Data are shown as the mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $***P < 0.0001$ using Student's *t* test.

In stark contrast with these data, IL-10 overexpression after the onset of the T cell response did not impact the outcome of infection. During the early stages of infection, IL-10 overexpression altered neither DC populations in MLN nor the kinetics of CD4⁺ T cell activation. Instead, CD4⁺ T cells primed in the IL-10–enriched environment were recruited to the lungs but accumulated in the vasculature and did not migrate into the parenchyma. This migratory deficit impaired antigen sensing, which limited the proliferation and effector cytokine production of recently activated effector CD4⁺ T cells. Crucially, the adoptive transfer of CD4⁺ T cells from the lungs of *M. tuberculosis*–infected mice into RAG$^{-/-}$ mice revealed that CD4⁺ T cells primed and differentiated in the IL-10–enriched environment maintain their vasculature-associated phenotype and are unable to restrain *M. tuberculosis* infection. Together our data support a model wherein IL-10 compromises the protective function of CD4⁺ T cells by promoting the differentiation of a vasculature-associated phenotype with reduced ability to translocate into the lung parenchyma and infiltrate the infected lesion.

Previous published data show that IL-10 inhibits cytokine production by monocytes (42) and macrophages (43) while downregulating MHC-II expression (40, 42). These effects of IL-10 are consistent with the reduced Th1 responses following *M. tuberculosis* infection observed in genetic (24, 26) and transgenic murine models of IL-10 overexpression (25). Our data show that this reduced Th1 response is a consequence of IL-10 production during the onset of the T cell response. This is consistent with a recent study showing that blockade of IL-10R signaling during the first 20 days after aerogenic *M. tuberculosis* infection reverts susceptibility in CBA/J mice (26).

Moreover, our data show that CD4⁺ T cells generated in an IL-10–enriched environment display an impaired parenchymal migratory potential. Likely associated with the reduced expression of CXCR3, this impaired potential ultimately hampers CD4⁺ T cells' protective function by compromising their ability to engage with infected phagocytes. Indeed, because T cells are better able to reduce bacterial growth if they are in direct contact with infected phagocytes (19), the parenchyma location of CD4⁺ T cells is crucial to control *M. tuberculosis* infection.

Early data on CXCR3$^{-/-}$ mice suggested a redundant role for this chemokine receptor in the recruitment of CD4⁺ T cells to the lungs of *M. tuberculosis*–infected mice (44). However, more recent data show

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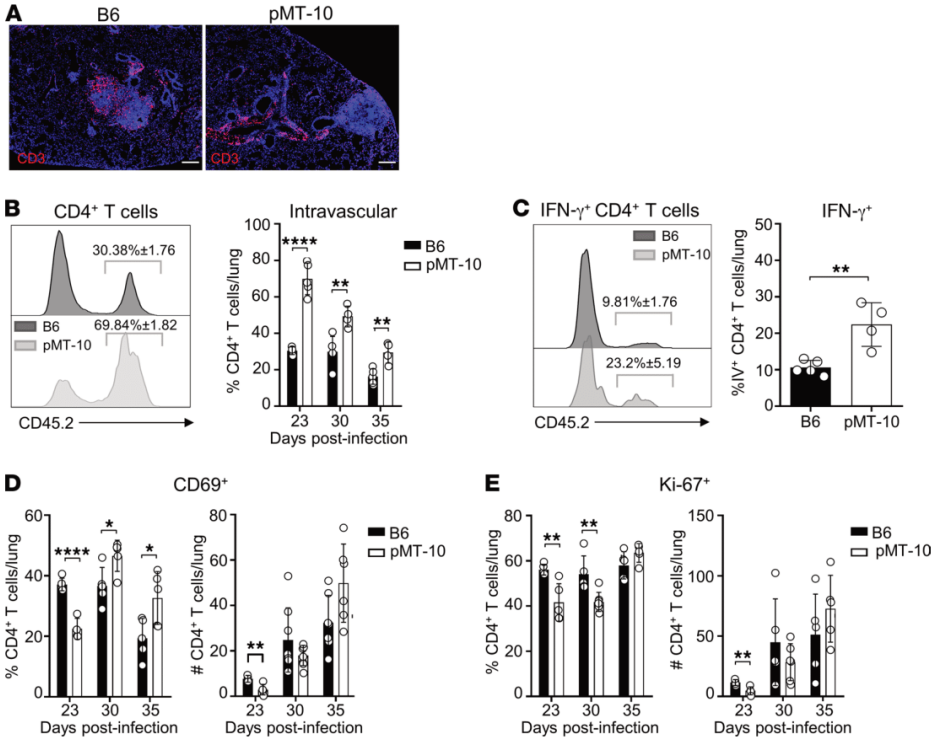

Figure 5. IL-10 overexpression limits CD4+ T cell migration into lung parenchyma and impairs antigen recognition and proliferation. B6 and pMT-10 mice were infected with *M. tuberculosis* H37Rv via aerosol route and IL-10 overexpression induced after day 5 after infection. (**A**) Representative immunofluorescence of CD3+ cells in lungs of mice at day 30 after infection. Scale bar: 200 μm. (**B**) Flow cytometry analysis and frequency of intravascular (CD45+) CD4+ T cells in lungs of mice throughout infection. (**C**) Flow cytometry analysis and frequency of intravascular (CD45+) IFN-γ–producing CD4+ T cells in lungs of mice at day 30 after infection. (**D**) Frequencies and numbers of CD69+CD4+ T cells from the lungs of mice throughout infection. (**E**) Frequencies and numbers of Ki-67+CD4+ T cells from lungs of mice throughout infection. Data represent 2 independent experiments with 4–5 mice per group. Data are shown as the mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ***$P < 0.0001$ using Student's *t* test.

that CXCR3 is crucial for CD4+ T cells to populate the lung parenchyma and induce control of *M. tuberculosis* growth (16–18). Indeed, deficiency in this chemokine receptor decreases by half the rate at which Th1 cells enter the lung parenchyma (41). Therefore, the reduced expression of CXCR3 seen on Ag-specific CD4+ T cells from pMT-10 mice overexpressing IL-10 in early stages of infection likely plays a crucial role in the delayed migration of these cells into the lung parenchyma.

In this regard, CXCR3 expression is rapidly upregulated early during differentiation of Th1 cells under Ag stimulation whereas the cytokine milieu has a minimal impact (45, 46). As such, the inhibition of cytokine production by IL-10 and, particularly, IL-12 (47), is likely to play a minor role in CXCR3 expression while the reduced expression of MHC-II during priming, as seen in this study, may have a more significant contribution. However, we anticipate that other chemokine receptors may also contribute to the migration of Ag-specific CD4+ T cells into the lung parenchyma, particularly in high IL-10 environments. Specifically, the expression of CCR2 and CXCR5 (and, to a lesser degree, CCR5 and CXCR6) has recently been shown to participate in Th1 cell recruitment to the lungs of *M. tuberculosis*–infected mice (41). Particularly important is the expression of the chemokine CXCL13 (48, 49) and that of its receptor CXCR5 (17, 50); both are crucially required for T cells to efficiently migrate into the lung parenchyma and into the infected lesion. While we did not see a negative impact of IL-10 in CXCL13, we did see a reduced expression of CXCR5 as well as B cell areas in the lungs of pMT-10 mice (B6 = 7.79 ± 3.50 vs. pMT-10 = 0.76 ± 0.22, $P \leq 0.05$).

The expression of indoleamine 2,3-dioxygenase (IDO1) has been shown to hamper the development of inducible bronchus-associated lymphoid tissue and to prevent the optimal homing of CD4+ T cells as well as

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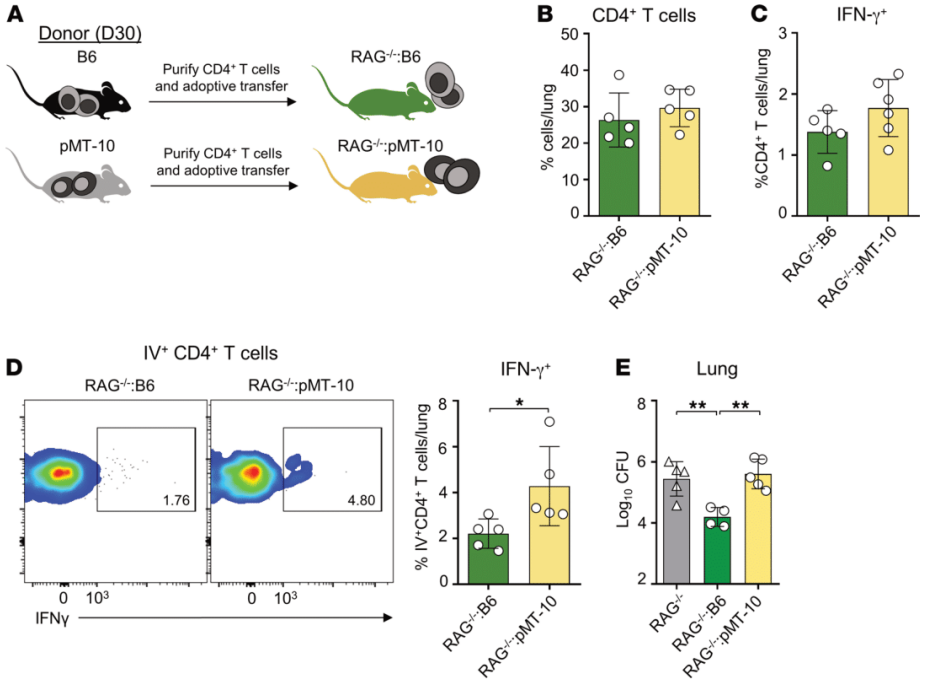

Figure 6. CD4+ T cells differentiated in IL-10–rich environment maintain their vasculature phenotype in an environment with normal levels of IL-10. (**A**) B6 and pMT-10 mice were infected with *M. tuberculosis* H37Rv via aerosol route and IL-10 overexpression was induced after day 5 after infection. At day 30 after infection, B6 and pMT-10 mice were sacrificed and 5 × 10^5 CD4+ T cells, purified from their lungs, were adoptively transferred into RAG-deficient mice infected 15 days earlier. Frequencies of (**B**) CD4+ T cells and (**C**) CD4+ T cells capable of producing IFN-γ in response to ESAT-$6_{1–20}$ at day 15 after adoptive transfer in lungs of infected mice. (**D**) Representative flow cytometry analysis and frequencies of CD4+ T cells capable of producing IFN-γ in response to ESAT-$6_{1–20}$ in lung vasculature of infected mice. Each flow plot represents 1 animal per group. (**E**) Lung bacterial burdens in RAG-/- mice that received B6 or pMT-10 effector cells at day 30 after infection (15 days after adoptive transfer). Data represent 2 independent experiments with 4–5 mice per group. Data are shown as the mean ± SD. *$P < 0.05$; **$P < 0.01$ using Student's *t* test.

their interaction with *M. tuberculosis*–infected phagocytes (51, 52). As the expression of IL-10 and that of IDO1 are linked (53), we questioned the impact of IDO1 in our model. However, we did not find increased expression of IDO1 in pMT-10 mice except at late time points after infection (Supplemental Figure 2). This suggests the activity of IDO1 is unlikely to contribute to the development of vasculature-associated CD4+ T cells seen in pMT-10 mice. On the other hand, IDO1 may contribute to the uncontrolled proliferation of *M. tuberculosis* at late stages of infection in pMT-10 mice with high levels of IFN-γ (Figure 2B) and with necrotic lesions (Figure 1C). However, this needs to be further addressed.

The developing consensus that protective CD4+ T cells locate in the lung parenchyma stems from studies showing that nonprotective antigen-specific T cells are associated with vasculature and express high KLRG1 and T-bet during chronic *M. tuberculosis* infection (16). KLRG1 expression marks a population of high cytokine-producing CD4+ T cells, particularly IFN-γ, during chronic *M. tuberculosis* infection, but not a self-renewing population. By contrast, PD-1 expression marks a population of self-renewing parenchymal CD4+ T cells (15). As the retention of CD4+ T cells in the vasculature of mice expressing high IL-10 is not a consequence of chronic antigen stimulation, we do not see increased KLRG1 expression. However, because progression of infection is associated with vasculature-associated CD4+ T cells, our data further support parenchymal migratory potential as a key feature of protective CD4+ T cells.

The fact that we see CD4+ T cells expanding in the lungs only after extensive bacterial proliferation occurs further reinforces the crucial role of the parenchymal migratory capacity of T cells for their response and protective function. As such, the data show that one of the crucial antagonistic effects of IL-10 in the control of *M. tuberculosis* infection is impeding the differentiation of effector CD4+ T cells with parenchymal migratory

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capacity. Accordingly, IL-10 overexpression after the onset of T cell responses does not influence the host's overall bacterial burden or disease pathology. We saw reduced frequency of $CD3^+CD4^+$ T cells (B6 = 80.33 ± 4.07 vs. pMT-10 = 73.09 ± 4.52, $P \leq 0.01$) and $CD4^+$ T cells capable of producing IFN-γ in response to $ESAT6_{1–20}$ (B6 = 3.47 ± 0.53 vs. pMT-10 = 2.9 ± 0.74, not significant) at day 65 after infection. However, this T cell reduction is likely a consequence of the IL-10 influence in the local environment of the lung lesion, as recently investigated (54). Moreover, it supports the limited contribution of IFN-γ–producing $CD4^+$ T cells in the control of *M. tuberculosis* infection in the lungs (55).

Although we do not see a negative impact of IL-10 during chronic infection, we do not exclude the possibility that further extending the overexpression of IL-10 throughout chronic infection would result in increased bacterial burdens, as other studies have suggested (25, 33, 39, 54, 56). However, the most likely explanation for this discrepancy is that IL-10 overexpression in our model is controlled by zinc supplementation; indeed, the levels of IL-10 are normal before zinc supplementation whereas, in other models, IL-10 is likely overexpressed throughout infection (25, 33). As such, these models' effects of IL-10 during the early stages of infection will affect bacterial burdens during chronic infection. Accordingly, the susceptible phenotype of CBA/J to *M. tuberculosis* infection is more clearly seen during chronic infection (24, 57); however, the blockade of IL-10 signaling during the first 21 days of infection was sufficient to revert the susceptible phenotype of CBA/J mice (26). These data further highlight the crucial role of IL-10 during the onset of the T cell response.

Previous data have shown that the early control of *M. tuberculosis* infection in BCG-vaccinated mice is not mediated by recently activated effectors, but likely by memory cells colonizing the lungs (58). Furthermore, blockade of IL-10R signaling during BCG vaccination has been shown to significantly increase protection against aerogenic challenge with *M. tuberculosis* (34). One key challenge in the rational design of new TB vaccines is to overcome the delayed activation and expression of antigen-specific responses in the lungs following aerosol challenge (59, 60). Therefore, together with results from earlier studies, our data suggest that targeting IL-10 or the IL-10 pathway during vaccination may help overcome the delayed T cell response and enhance vaccine-induced protection.

Together our data demonstrate that IL-10 antagonizes the control of pulmonary *M. tuberculosis* infection by impeding the differentiation of T cells with capacity to migrate into lung parenchyma. These data, when taken together with other recent data showing enhanced vaccine-induced protection through IL-10 blockade during BCG vaccination and after *M. tuberculosis* infection (34), suggest that IL-10 may be an impeding factor to the rapid expression of T cell immunity in the lungs. Future vaccines should promote the development of T cells capable of rapidly migrating, of persisting within the lung parenchyma, and of colocating with infected phagocytes.

Methods

Mice. C57BL/6 and B6.129S7-Rag1^{tm1Mom}/J ($RAG^{-/-}$) mice were bred at the ICVS animal facility from stock purchased from Charles River Laboratories and the Jackson Laboratory, respectively. Breeders of P25 TCR transgenic mice that recognize Peptide-25 of the immunodominant antigen Ag85b of *M. tuberculosis*, presented in the context of $I\text{-}A^b$, were originally obtained from Anne O'Garra's lab at the Francis Crick Institute, London, United Kingdom; these were previously described (61). pMT-10 mice on a C57BL/6 background were produced by António G. Castro and Paulo Vieira, as previously described (35). Briefly, mouse IL-10 cDNA sequence was cloned in the p169ZT vector, which carries the sheep metallothionein (MT) Ia promotor, a β-globin splice site and the SV40 polyadenylation signal. The resulting vector was injected in C57BL/6 eggs and transgenic founders were identified by PCR using MT-specific primers. IL-10 overexpression in pMT-10 mice was induced by supplementing their drinking water with 2% sucrose solution containing 50 mM of zinc sulphate, as previously described (35). The resulting rapid increase in circulating levels of IL-10 were maintained until zinc sulphate was withdrawn (35). B6 mice used as controls were maintained in the same condition as pMT-10 mice, including drinking water supplemented with 2% sucrose solution containing 50 mM of zinc sulphate. Both male and female mice between the ages of 6 and 12 weeks were used for experimental procedures.

M. tuberculosis aerosol infection and bacterial load determination. The H37Rv strain of *M. tuberculosis*, originally from the Trudeau Institute, was used in this study. To generate *M. tuberculosis*–mCherry, the parental *M. tuberculosis* strain was transformed using the plasmid pBP10 provided by David G. Russel, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA, as described previously (62, 63). Both *M. tuberculosis* and *M. tuberculosis*–mCherry were grown to log

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phase in Middlebrook 7H9 broth supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.2% glycerol, 0.05% Tween 80, and 50 μg/ml hygromycin B (for *M. tuberculosis*–mCherry), and subcultured in Proskauer Beck medium with 0.05% Tween 80 to mid-log phase before frozen at −80°C. These frozen stocks were quantified and used to infect mice with a low dose of bacteria (~75 CFUs) using a Glas-Col airborne infection system, as previously described (18). At selected time points after challenge, mice were killed by CO_2 asphyxiation; organs were aseptically excised and individually homogenized in saline. Organ homogenates were then 10-fold serial-diluted and plated on nutrient 7H11 agar (BD Biosciences) for 3 weeks at 37°C, at which point CFUs were counted.

Sample collection and preparation of lung single-cell suspensions. Aseptically excised lungs were sectioned and incubated at 37°C for 30 minutes with collagenase D (0.7 mg/mL, Sigma-Aldrich). Lungs were then disrupted into a single-cell suspension by passage through a 70 μm nylon cell strainer (BD Biosciences). After centrifugation, the cell-free suspensions were aliquoted and frozen at −80°C until their concentrations of cytokines were determined using ELISA kits (Thermo Fisher Scientific), following the manufacturer's instructions. Lung single-cell suspensions were then treated with erythrocyte lysis buffer (0.87% of NH_4Cl). To remove cell debris and nonhematopoietic cell interference, lung cells were further processed over a 40:80% Percoll (GE Healthcare). The resulting cell suspension was washed twice and counted. A single-cell suspension was prepared from the MLNs by passing the organs through a 70 μm nylon cell strainer (BD Biosciences), followed by treatment with erythrocyte lysis buffer.

For intravital flow cytometry analysis, mice received APC-labeled CD45 antibody intravenously 3 minutes before euthanasia (16). Lung or MLN single-cell suspensions were then stained with fluorochrome-conjugated antibodies for 30 minutes on ice. For tetramer staining, cells were incubated in the dark for 1 hour at 37°C with the Brilliant Violet 421–conjugated I-A^b ESAT-$6_{4–17}$ tetramer provided by the NIH Tetramer Core Facility. For intracellular cytokine detection, cells were cultured in 5 μg/ml of ESAT-$6_{1–20}$ peptide for 1.5 hours before 10 μg/ml Brefeldin A (Sigma-Aldrich) was added to the culture for an additional 3.5 hours. The cells were phenotyped using the following antibodies: CD3 Brilliant Violet 605 (145-2C11, BioLegend), CD4 APC-Cy7 (GK1.5, BioLegend), CD11b PE-Cy7 (M1/70, BioLegend), CD11c Brilliant Violet 650 (N418, BioLegend), Ly-6C PerCPCy5.5 (HK1.4, BioLegend), MHC-II FITC (M5/114.15.2, BioLegend), CD86 APC (GL-1, BioLegend), CD44 PerCPCy5.5 (IM7, BioLegend), CD69 PE (H1.2F3, eBioscience), CD62L PE/Cy7 (MEL-14, BioLegend), CD45 Brilliant Violet 510 (30-F11, BioLegend), CD45.2 APC (104, BioLegend), Ki-67 PE/Cy7 (SoIA15, eBioscience) and IFN-γ PE/Cy7 (XMG1.2, BioLegend). Data were acquired on an LSR II flow cytometer (BD Biosciences) using Diva software and analyzed using FlowJo software (BD Biosciences). The total number of cells for each population was determined based on the percentage of cells measured by flow cytometry and the total number of cells per lung.

$CD4^+$ T cell adoptive transfers. For adoptive transfers of P25 TCR-Tg $CD4^+$ T cells, the spleens of P25 mice were collected and processed into single-cell suspensions. Naive $CD4^+$ T cells were negatively selected using the Naive $CD4^+$ T Cell Isolation Kit from Miltenyi Biotec, following the manufacturer's instructions. Purified cells were then stained with 5 μM CFSE (Molecular Probes) for 10 minutes at 37°C and adoptively transferred into recipient B6 and pMT-10 mice (1×10^6 total $CD4^+$ T cells per mouse). The following day, B6 and pMT-10 recipients were sacrificed and their MLNs analyzed for the presence of $CFSE^+$ (P25TCR-tg) transferred cells.

For adoptive transfer of effector $CD4^+$ T cells into $RAG^{-/-}$ mice, the lungs of B6 and pMT-10 mice were harvested at day 28 after *M. tuberculosis* infection and processed into single-cell suspensions, as described above. $CD4^+$ T cells were magnetically labeled with CD4 (L3T4) microbeads (Miltenyi Biotec) and purified following the manufacturer's instructions. Positively selected lung $CD4^+$ T cells were then counted and injected intravenously into $RAG^{-/-}$ mice infected with *M. tuberculosis* 15 days earlier (5×10^5 cells per mouse). At day 30 after infection (15 days after adoptive transfers), the lungs were aseptically excised to determine bacterial loads.

Anti–IL-10R mAb treatment. One day prior to *M. tuberculosis* infection, mice were injected i.p. with 1 mg of either anti–IL-10 receptor (IL-10R) mAb (CD210) or IgG1 isotype control mAb (both from Bio X Cell), as previously described (64). Mice were then infected with *M. tuberculosis* through the aerosol route. To maintain IL-10R blockade, mice received 0.35 mg of the respective mAb i.p. at weekly intervals until the designated experimental times.

Histology and immunohistochemistry. The upper right lobe of each lung was inflated with 4% PFA and processed routinely for light microscopy with hematoxylin and eosin stain. Morphometric analysis was

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performed in a blinded manner using ImageJ software (version 1.50e; NIH). The percentage of total lung area involved with inflammation was calculated by dividing the cumulative area of inflammation by the total lung surface area for each sample.

Immunofluorescence was performed on formalin-fixed lung sections as described previously (65). Sections were probed using a purified rabbit polyclonal anti-CD3e (1:100; ab185811, Abcam), and visualized by adding Alexa Fluor 568 goat anti-rabbit (1:500; A-11011, Invitrogen). SlowFade Gold Antifade Mountant with DAPI (Invitrogen) was used to counterstain tissues and to detect nuclei. Representative images were obtained using an Olympus BX61 microscope and were recorded using a digital camera (DP70, Olympus) using the Olympus cell^P software.

Quantitative RT-PCR analysis. Quantitative RT-PCR (qRT-PCR) was performed as previously described (66). Total RNA from whole lungs was extracted using TRIzol (Invitrogen) from which cDNA was generated using the GRS cDNA Synthesis Mastermix (Grisp), following the manufacturer's instructions. The resultant cDNA template was used to quantify the expression of target genes by qRT-PCR (CFX96 Real-Time System with C1000 Thermal Cycler, Bio-Rad) and normalized to ubiquitin mRNA levels using the ΔCt method. Target gene mRNA expression was quantified using SYBR Green (Thermo Fisher Scientific) and specific oligonucleotides (Invitrogen).

Statistics. Differences between groups were analyzed using a 2-tailed unpaired Student's *t* test or 1-way ANOVA as appropriate. Survival curves were analyzed using the log-rank test. Data were displayed as mean ± SD. Differences were considered significant when $P \leq 0.05$.

Study approval. All procedures involving live animals were performed in accordance with the European Directive 86/609/EEC and approved by the Subcomissão de Ética para as Ciências da Vida e da Saúde (SECVS 074/2016) (University of Minho, Braga, Portugal) and the Portuguese National Authority Direcção Geral de Alimentação e Veterinária (DGAV 014072) (Lisbon, Portugal).

Author contributions

CMF, AGC, and ET conceived and designed the study. CMF, AMB, and PBS performed the experimental work and data analysis. RS, CC, AC, FR, and MCN provided technical and/or material support. CMF, AGC, and ET drafted the manuscript. AGC and ET acquired funding. All authors critically revised and approved the manuscript and accepted accountability.

Acknowledgments

We thank the personnel of the ICVS animal facility and histology core for all their technical assistance. This work was supported by national funds through the Foundation for Science and Technology (FCT) projects PTDC/SAU-INF/28463/2017, PTDC/MED-ONC/28658/2017, UIDB/50026/2020, and UIDP/50026/2020 and by the Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) projects NORTE-01-0145-FEDER-000013 and NORTE-01-0145-FEDER-000023. ET and RS were supported by the FCT Investigator grants IF/01390/2014 and IF/00021/2014, respectively; AC and CC by Estímulo Individual ao Emprego Científico CEECIND/03628/2017 and CEECIND/04058/2018, respectively; and CMF and AMB by FCT PhD fellowships PD/BD/137447/2018 and SFRH/BD/120371/2016, respectively.

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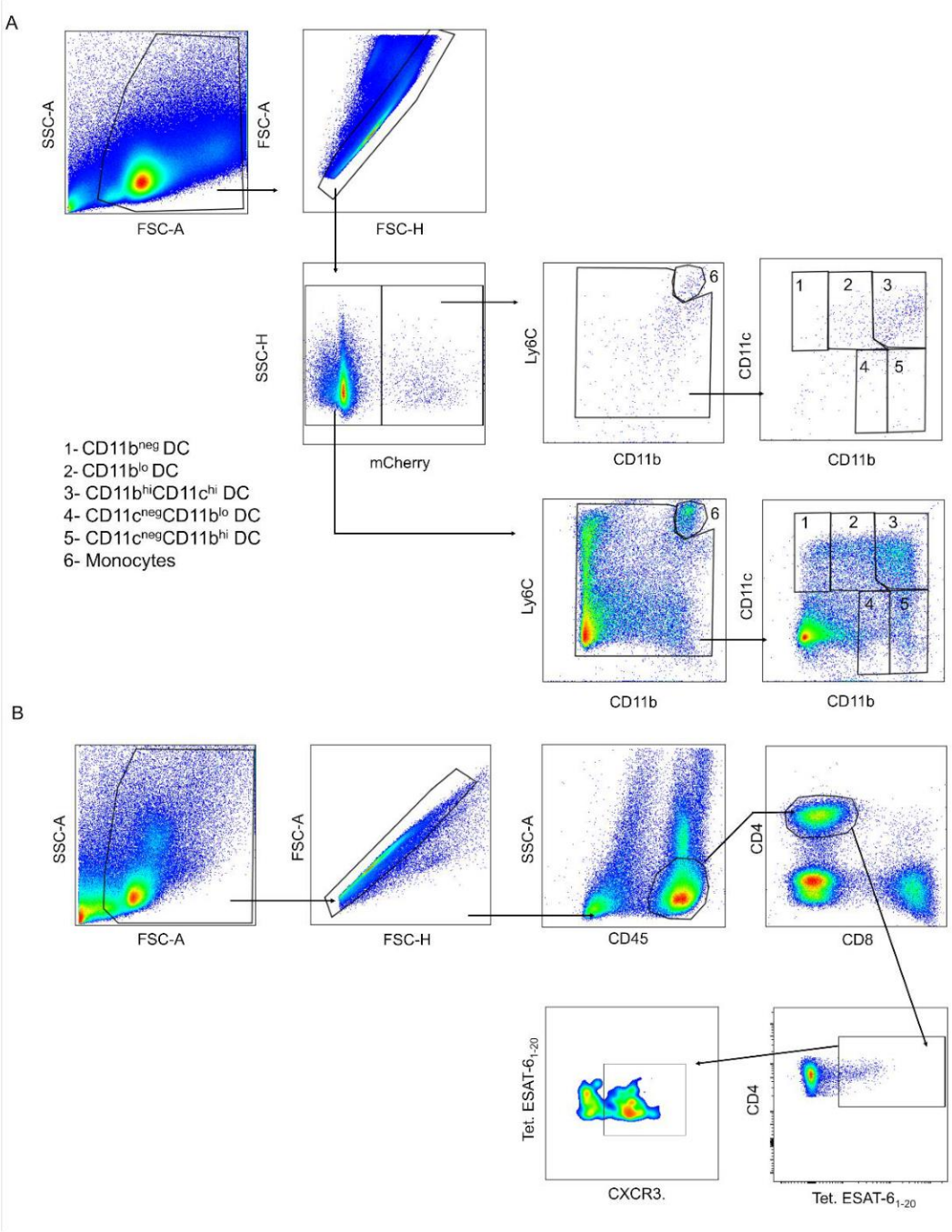
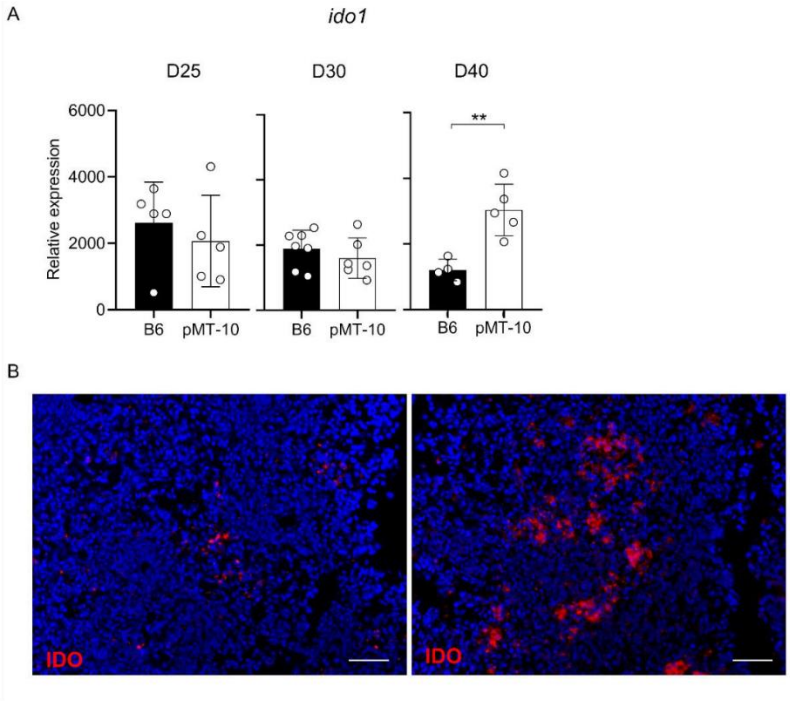
Supplementary Data



Supplemental Figure 1- Gating strategy used to identify (A) myeloid cell populations in the mLN, and (B) CXCR3 expression by I-A^b ESAT-6$_{4-17}$-specific CD4$^+$ T cells in the lungs of Mtb-infected mice.



Supplemental Figure 2- Increased expression of indoleamine 2,3-dioxygenase (IDO1) in pMT-10 mice at the very late stages of Mtb infection. B6 and pMT-10 mice were infected with Mtb H37Rv via aerosol route and IL-10 overexpression was induced after day 5 post-infection. **(A)** Relative expression of *Ido1* in the lungs of infected mice at days 25, 30 and 40 post-infection. **(B)** Representative immunofluorescence staining of IDO1+ cells in the lungs of mice at day 40 post-infection. Sections were probed with a purified rabbit polyclonal anti-IDO antibody (1:100; PA5-24598, ThermoFisher Scientific), and visualized by adding Alexa Fluor 568 goat anti-rabbit (1:500; A-11011, Invitrogen). Scale bar represents 50µm. Data are representative of 2 independent experiments with 4-5 mice per group. Error bars represent the mean ± SD. **, $P < 0.01$ using student's t-test.

Chapter III

BCG vaccination prevents the fast progression of *Mycobacterium tuberculosis* infection induced by IL-10

BCG vaccination prevents the fast progression of *Mycobacterium tuberculosis* infection induced by IL-10

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Manuscript in preparation.
CMF contributed to all figures of this work.

Abstract

Control of tuberculosis depends on the rapid expression of protective $CD4^+$ T cell responses in the *Mycobacterium tuberculosis* (Mtb) infected lungs. We have recently shown that the immunomodulatory cytokine IL-10 acts intrinsically in $CD4^+$ T cells and impairs their parenchymal migratory capacity thereby preventing control of Mtb infection. Herein, we show that IL-10 does not impact the protection conferred by the established memory T cell response, as BCG-vaccinated mice overexpressing IL-10 only during Mtb infection display an accelerated Ag85b-specific $CD4^+$ T cell response and improved control of Mtb infection. Despite this, our data show that IL-10 inhibits the migration of ESAT-6-specific $CD4^+$ T cells into the lung parenchyma and impairs the development of ectopic lymphoid structures associated with reduced expression of the chemokine receptors CXCR5 and CCR7. Together, our data supports a role for BCG vaccination in preventing the immunosuppressive effects of IL-10 in the fast progression of Mtb infection and may provide valuable insights on the mechanisms contributing to the variable efficacy of BCG vaccination.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is still a leading cause of infectious death worldwide second only to COVID-19 (1). The COVID-19 pandemic reversed years of global progress in reducing the number of people who die from TB. Indeed, due to the COVID-19 pandemic there has been a significant decline in TB notification rates and interventions; this reduction has caused, for first time in more than a decade, an increase in the number of TB deaths (1). These epidemiological changes are furthermore expected to have serious consequences for the global control of TB in the coming years. Indeed, an excess of 1.4 million TB deaths and 6.3 million cases of active TB are estimated to occur by 2025 (1).

Vaccination is the most sustainable and cost-effective answer to this threat. However, the only licensed TB vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), while effective in preventing disseminated forms of pediatric TB is not efficient against adult pulmonary disease (2–4). This limited protection against pulmonary disease is, in part, a consequence of the short-lived immunity conferred by BCG vaccination, which typically wanes by adolescence (5). However, there is now revived optimism in BCG revaccination to control pulmonary TB in adolescents and adults, with a recent study showing an efficacy of BCG revaccination of 45.4% against sustained Mtb infection (6). However, despite these promising results, vaccines with improved efficacy of >50% are considered necessary to reach the target of the WHO End TB strategy (7). It is therefore critical to understand why BCG is ineffective against pulmonary disease in order to devise strategies to improve its efficacy and the efficacy of novel vaccines.

Interleukin-10 (IL-10) has been implicated in increased susceptibility to TB in both humans and animal models, an event highly associated with decreased $CD4^+$ T cell responses (8–13). Using a mouse model of controlled IL-10 overexpression, we have recently demonstrated that IL-10 overexpression before the onset of the acquired immune response promotes the differentiation of vasculature-associated $CD4^+$ T cells with reduced ability to migrate to the lung parenchyma and infiltrate the infected lesion (14). That this is a central mechanism whereby IL-10 antagonizes control of Mtb infection is demonstrated by our data showing that IL-10 overexpression after the onset of the T cell response does not significantly impact control of infection (14). As such, the parenchymal migratory capacity and ability to engage with infected phagocytic cells are critical features of protective T cells. Indeed, the parenchyma location of $CD4^+$ T cells is crucial to control Mtb infection, as T cells are better able to reduce bacterial growth by directly interacting with infected phagocytes (15). Furthermore, the accumulation of $CD4^+$ T cells in the lung parenchyma promotes the development of a more protective granuloma characterized by the presence of organized B cell aggregates, reminiscent of ectopic lymphoid structures (16). Indeed, the formation of

these structures has been associated with control of infection in animal models (17–19) and with controlled latent TB in humans (16, 20). While B cell follicle formation by itself is not protective in the mouse model of TB, the cytokines and chemokines associated with the development of these cellular aggregates are critical for T cell localization within the lung and ability to engage with infected phagocytes to induce Mtb control (16, 21, 22). In this regard, the expression of CCL19, CCL21 and CXCL13 and their receptors, CCR7 and CXCR5, have been shown to be crucial for the formation of ectopic lymphoid structures and control of Mtb infection (16, 22, 23).

As we recently demonstrated that IL-10 antagonized the control of Mtb infection by driving the differentiation of $CD4^+$ T cells with reduced parenchyma migratory capacity; in this work, we sought to determine if the impact of IL-10 in the $CD4^+$ T cell response and control of infection is similar in vaccinated mice as we previously reported in naive mice (14). To this end, we used our mouse model of controlled IL-10 overexpression (pMT-10) (14, 24) which were vaccinated with BCG for two months before infection with Mtb through the aerosol route. We then induced IL-10 overexpression and determined the ability of pMT-10 mice to mount a protective response and control the infection. Our data show that BCG vaccination prevents early disease progression induced by IL-10 overexpression in unvaccinated mice, associated with an early accumulation of Ag85b-specific $CD4^+$ T cells in the lungs. Despite this, BCG vaccination does not overcome the deficient migration of ESAT-6-specific $CD4^+$ T cells into the lung parenchyma previously described in naïve mice (14). Importantly, despite the improved control of infection there is a reduced formation of B cell aggregates in the lungs of BCG-vaccinated pMT-10 mice. These data show that IL-10 overexpression has a differential impact in the primary and secondary responses. These findings may be relevant to unravel new mechanisms contributing to the variable efficacy of BCG vaccination in preventing pulmonary TB, particularly in individuals with intrinsically higher levels of IL-10.

2. Methods

2.1. Mice.

B6 mice were bred at the ICVS animal facility from stock purchased from Charles River Laboratory (Barcelona, Spain). pMT-10 mice on a C57BL/6 background were produced by Drs. António G. Castro and Paulo Vieira, as previously described (24). Briefly, mouse IL-10 cDNA sequence was cloned in the p169ZT vector, which carries the sheep metallothioneinc (MT) Ia promotor, a β-globin splice site and the SV40 polyadenylation signal. The resulting vector was injected in C57BL/6 eggs and transgenic founders were identified by PCR using MT specific primers. IL-10 overexpression in pMT-10 mice was induced by supplementing the drinking water with 2% sucrose solution containing 50 mM of zinc sulphate, as previously described (24). This results in a rapid increase in the circulating levels of IL-10, which are maintained until zinc sulphate withdraw (24). B6 mice used as controls were maintained in the same condition as pMT-10 mice including drinking water supplemented with 2% sucrose solution containing 50 mM of zinc sulphate. Both male and female mice between the ages of 6 and 12 wk old were used for experimental procedures. All procedures involving live animals were performed in accordance with the European Directive 86/609/EEC and approved by the *Subcomissão de Ética para as Ciências da Vida e da Saúde* (SECVS 074/2016) and the Portuguese National Authority *Direcção Geral de Veterinária* (DGAV 014072).

2.2. BCG vaccination, Mtb aerosol infection and bacterial load determination.

Mice were vaccinated with 1×10^6 *M. bovis* BCG Pasteur via the subcutaneous route for 60 days prior to Mtb aerosol infection. The H37Rv strain of Mtb, originally from the Trudeau Institute (Saranac Lake, NY), was grown to log phase in Middlebrook 7H9 broth supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC), 0.2% glycerol, 0.05% Tween 80, and sub-cultured in Proskauer-Beck medium with 0.05% Tween-80 to mid-log phase before frozen at -80°C. These frozen stocks were quantified and used to infect mice with a low dose of bacteria (~75 CFUs) using a Glas-Col airborne infection system, as previously described (14). At selected time points after challenge, mice were killed by CO_2 asphyxiation and the organs were aseptically excised and individually homogenized in saline. Organ homogenates were then 10-fold serial diluted and plated on nutrient 7H11 agar (BD Biosciences) for 3 wk at 37ºC, at which point CFUs were counted.

2.3. Sample collection and preparation of lung single cell suspensions.

Aseptically excised lungs were sectioned and incubated at 37ºC for 30 min with collagenase D (0.7mg/mL, Sigma). Lungs were then disrupted into a single cell suspension by passage through a 70-

μm nylon cell strainer (BD Biosciences). Lung single cell suspensions were then treated with erythrocyte lysis buffer (0.87% of NH_4Cl). To remove cell debris and non-hematopoietic cell interference, lung cells were further processed over a 40:80% Percoll (GE Healthcare). The resulting cell suspension was washed twice and counted.

For intravital flow cytometry analysis, mice received APC-labelled anti-CD45 antibody intravenously 3 min before euthanasia (28). Lung single cell suspensions were then stained with fluorochrome-conjugated antibodies for 30 min on ice. For intracellular cytokine detection, cells were cultured in 5 μg/ml of $Ag85b_{240-254}$ or $ESAT\text{-}6_{1-20}$ peptide for 1.5 h before 10 μg/ml Brefeldin A (Sigma-Aldrich) was added to the culture for 3.5 h more. Cells were phenotyped by flow cytometry using the following antibodies: CD3-Brilliant Violet 605 (145-2C11, BioLegend), CD4-APC-Cy7 (GK1.5, BioLegend), CD44-PercPCy5.5 (IM7, BioLegend), CD45-Brilliant Violet 510 (30-F11, Biolegend), CD45.2-APC (104, BioLegend), and IFNγ-PE-Cy7 (XMG1.2, BioLegend). Data were acquired on a LSRII flow cytometer (BD Biosciences) with Diva Software and analyzed using FlowJo software (BD Biosciences). The total number of cells for each population was determined based on the percentage of cells determined by flow cytometry and the total number of cells per lung.

2.4. Histology and Immunohistochemistry.

The right upper lobe of each lung was inflated with 4% paraformaldehyde (PFA) and processed routinely for light microscopy with hematoxylin and eosin stain. Morphometric analysis was performed in a blinded manner using ImageJ software (version 1.50e; NIH). The percentage of total lung area involved with inflammation was calculated by dividing the cumulative area of inflammation by the total lung surface area for each sample. The percentage of B cell aggregation in the granuloma was calculated by dividing the area of lung tissue stained positive for B220 by the area of inflammation in the lung.

Immunofluorescence was performed on formalin-fixed lung sections as described previously (14). Sections were probed with either purified rabbit polyclonal anti-CD3e (ab185811; Abcam) or CD45R (RA3-6B2; Abcam) and visualized by adding streptavidin–Alexa Fluor 488 (Invitrogen). SlowFade Gold antifade with DAPI (Invitrogen) was used to counterstain tissues and to detect nuclei. Representative images were obtained with an Olympus BX61 microscope and were recorded with a digital camera (DP70) using the cell^P software.

2.5. Real-time (RT) PCR analysis

Total RNA from infected lungs was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. cDNA was generated from 1 ug of RNA using the GRS cDNA Synthesis Master Mix (Grisp)

following the manufacturer's instructions. The resultant cDNA template was used to quantify the expression of target genes by RT-PCR (Bio-Rad CFX96 Real-Time System with C1000 Thermal Cycler) using the following protocol: one cycle of 95oC for 3 minutes, followed by 40 cycles of a two-stage temperature profile of 95ºC for 3 seconds and 60ºC for 30 seconds. Gene expression was normalized to Ubiquitin mRNA levels using the ΔCt method. Target gene mRNA expression was quantified using SYBR green (Thermo Scientific) and specific oligonucleotides (Table 1; Invitrogen, Carlsbad, CA).

Table 1- Sequence of primers used to determine gene expression by RT-PCR

Target	Forward Sequence	Reverse Sequence
Ubq	TGGCTATTAATTATTCGGTCTG	GCAAGRGGCTAGAGTGCAGAGTA
Ccl19	CCTGCTGTTGTGTTCACCACA	TGTTGCCTTTGTTCTTGGCA
Ccl21	TCCAACTCACAGGCAAAGAGG	GGCCGTGCAGATCTAATGGTT
Cxcl13	CTCCAGGCCACGGTATTCTG	CCAGGGGGCGTAACTTGAAT
Ccr7	GTACGAGTCGGTGTGCTTC	GGTAGGTATCCGTCATGGTCTTG
Cxcr5	CAGACTTCATGCCAGACCTTCA	CCAATGCTGGTTAGGTTGGAAC

2.6. Statistical Analysis.

Differences between groups were analyzed using one-way ANOVA. Differences were considered significant when $p \leq 0.05$ and denoted as *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ and ****, $p \leq 0.0001$.

2.7. Acknowledgements

We thank the personnel of the ICVS animal facility and histology core for all the technical assistance. This work was supported by National funds through the Foundation for Science and Technology (FCT) - projects PTDC/SAU-INF/28463/2017, PTDC/MED-ONC/28658/2017, UIDB/50026/2020 and UIDP/50026/2020; and by the Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) projects NORTE-01-0145-FEDER-000013 and NORTE-01-0145-FEDER-000023. ET, RS and CC were supported by the FCT Estímulo Individual ao Emprego Científico 2020.03070.CEECIND, 2020.00185.CEECIND, and 2018.04058.CEECIND, respectively; and CMF, CM and AMB by FCT PhD fellowships PD/BD/137447/2018, 2020.05976.BD, and SFRH/BD/120371/2016, respectively.

3. Results

3.1. BCG vaccination prevents the fast progression of Mtb infection induced by IL-10.

Our previous data demonstrated that $CD4^+$ T cells differentiated in an IL-10 enriched environment differentiated into a vasculature-associated phenotype with reduced ability to migrate into the parenchyma and infiltrate the Mtb lung lesion (14). This migratory deficit prevented Mtb control, exacerbating bacterial proliferation and pathology (14). As early protection conferred by BCG vaccination has been shown to depend on lung-resident memory $CD4^+$ T cells (25), we questioned the impact of IL-10 in vaccinated mice. To answer this, we vaccinated B6 and pMT-10 mice with $1x10^6$ BCG through the subcutaneous route. Two months after vaccination, mice were infected with Mtb through the aerosol route, and the expression of IL-10 in pMT-10 mice activated by supplementing the drinking water with 50 mM of zinc sulphate, as previously described (14, 24). B6 mice maintained in the same conditions as pMT-10 mice were used as controls.

As expected, our data showed that BCG vaccinated B6 mice were more protected against Mtb infection than unvaccinated B6 mice, with 1.5 Log_{10} less bacterial burdens in the lungs at day 30 post-infection (Figure 1A). In line with our previous study (14), pMT-10 mice exhibited exacerbated lung bacterial burdens when compared to B6 mice (Figure 1A). Interestingly however, BCG-vaccinated pMT-10 mice displayed significantly lower lung bacterial burdens than control pMT-10 mice. Indeed, bacterial proliferation in the lungs of vaccinated pMT-10 mice was similar to control B6 mice (Figure 1A). Accordingly, our data show that both B6 and pMT-10 vaccinated mice present fewer areas of lesioned lung tissue than control animals (Figure 1B). Together, these data show that BCG vaccination prevents the acute susceptibility to Mtb infection induced by IL-10, allowing for improved control of bacterial proliferation and pathology.

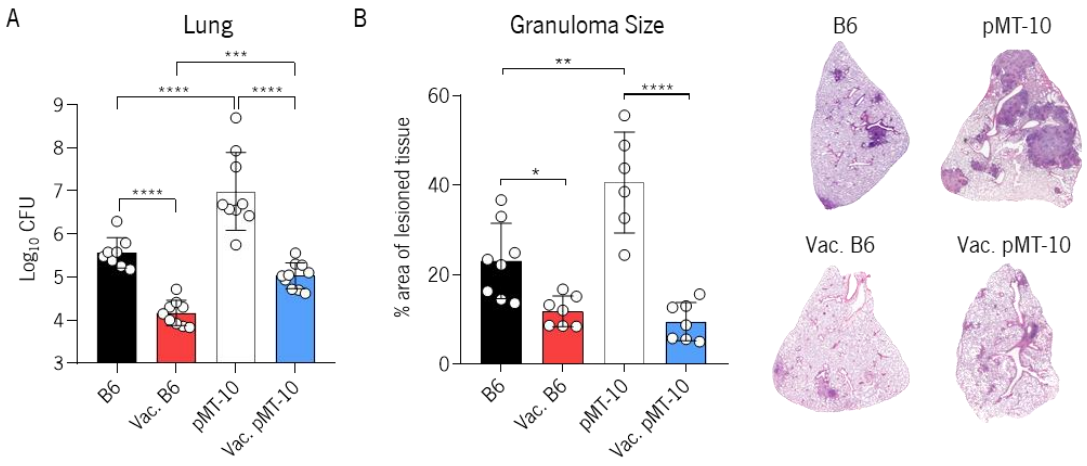

Figure 1- BCG vaccination protects pMT-10 mice from bacterial proliferation and lung pathology. B6 and pMT-10 mice were vaccinated with1x10^6 BCG for 60 days prior infection with Mtb H37Rv via aerosol route. IL-10 overexpression was induced 5 days after infection. Unvaccinated mice were used as controls. **(A)** Lung bacterial burdens and **(B)** representative hematoxylin and eosin-stained lung sections and percentage of infiltrated area in the lungs at day 30 post-infection. Individual data points represent individual animals. Data are representative of 3 independent experiments with 5 mice per group. *p< 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by one-way ANOVA followed by Tukey's test.

3.2. IL-10 does not impair the BCG-induced Ag85b-specific CD4$^+$ T cell response following Mtb challenge.

To determine whether the increased protection of vaccinated mice overexpressing IL-10 correlated with an improved CD4$^+$ T cell response, we first analyzed the accumulation of CD4$^+$ T cells expressing the activation marker CD44 in the lungs at days 15 and 30 post-infection. We found an increased accumulation of CD4$^+$CD44$^+$ T cells in the lungs of vaccinated mice at day 15 post-infection than in unvaccinated mice (Figure 2A). However, by day 30 post-infection, the frequency and number of CD4$^+$CD44$^+$ T cells were similar across all groups of mice (Figure 2A). To determine further the impact of BCG vaccination in the development of the Ag-specific response in the IL-10-enriched environment, we evaluated the frequency and numbers of Ag85b-specific CD4$^+$ T cells and their ability to produce IFN-γ. To do this, we restimulated lung cells with Ag85b and quantified IFN-γ-producing CD4$^+$ T cells at 15 and 30 days after Mtb infection (Figure 2B). Our data show that both groups of vaccinated mice presented increased frequency and number of Ag85b-specific IFN-γ-producing CD4$^+$ T cells when compared to control mice at day 15 post-infection (Figure 2B). Importantly, the accumulation of Ag85b-specific IFN-γ-producing CD4$^+$ T cells in the lungs of vaccinated pMT-10 mice appears to occur in a similar proportion as in vaccinated B6 mice, suggesting a minor role for IL-10 in the development of this response after BCG vaccination (Figure 2B). By day 30 post-infection, the frequency and number of CD4$^+$ T cells producing IFN-γ in response to Ag85b were similar across all groups of mice (Figure 2B). These results support previous evidence that BCG-vaccinated mice exhibit an accelerated Ag85b-specific CD4$^+$ T cell

response when compared to unvaccinated mice (26). Additionally, these data demonstrate that IL-10 overexpression during infection does not influence the BCG-mediated accumulation of Ag85b-specific $CD4^+$ T cells early following Mtb challenge.

Our previous study revealed that IL-10 delayed the onset of the ESAT-6-specific $CD4^+$ T cell response. Therefore, we decided to explore the impact of IL-10 expression in the development of ESAT-6-specific $CD4^+$ T cell response in BCG vaccinated mice. To do this, we analyzed the frequency and number of IFNγ-producing $CD4^+$ T cells in response to ESAT-6 restimulation at days 15 and 30 post-infection. We found a similar frequency and number of ESAT-6-specific IFN-γ-producing $CD4^+$ T cells in the lungs of all experimental groups by day 15 post-infection. However, at day 30 after infection, the frequency of ESAT-6-specific IFN-γ-producing $CD4^+$ T cells in the lungs of both unvaccinated B6 and pMT-10 mice were higher than in BCG vaccinated B6 and pMT-10 mice (Figure 2C). This decreased expansion is likely a consequence of the lower lung bacterial burdens and the stronger Ag85b-specific $CD4^+$ T cells response already taking place in these mice at day 15 post-infection (Figure 2B).

Altogether, these data demonstrate that the onset of the BCG-specific $CD4^+$ T cell response is similar in both B6 and pMT-10 vaccinated mice, thus supporting the hypothesis that IL-10 overexpression during infection does not impair BCG-mediated immunity.

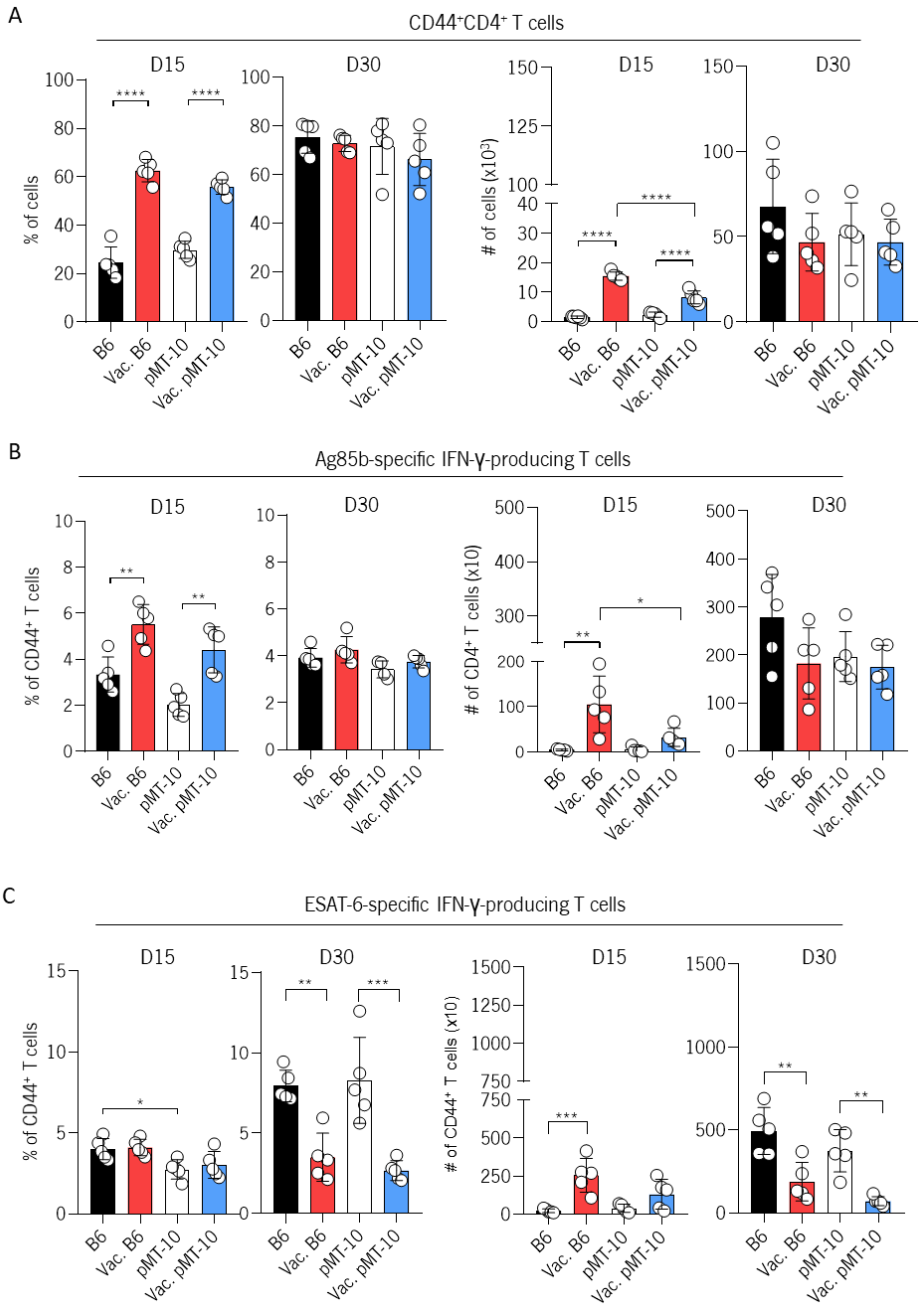

Figure 2- IL-10 expression does not influence CD4⁺ T cell accumulation in the lungs of vaccinated mice. B6 and pMT-10 mice were vaccinated with1x10⁶ BCG for 60 days prior infection with Mtb H37Rv via aerosol route. IL-10 overexpression was induced 5 days after infection. Unvaccinated mice were used as controls. **(A)** Frequencies and numbers of CD4⁺ T cells expressing CD44 in the lungs of mice at days 15 and 30 post-infection. **(B)** Frequencies and numbers of IFN-γ–producing CD4⁺ T cells after in vitro restimulation with the Ag85b peptide. **(C)** I Frequencies and numbers of IFN-γ–producing CD4⁺ T cells after in vitro restimulation with the ESAT-6$_{1-20}$ peptide. Data represent 2 independent experiments with 5 mice per group. *p< 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA followed by Tukey's test.

3.3. BCG vaccination does not prevent the IL-10-dependent accumulation of CD4⁺ T cells in the lung vasculature.

Our previous study revealed that IL-10 antagonized control of Mtb infection by promoting the generation of vasculature-associated CD4⁺ T cells with reduced ability to migrate into the lung parenchyma

and induce control of infection (14). In the present study, we showed that BCG vaccination prevents the antagonistic effects of IL-10 likely because of memory lymphocytes that are retained in the lung and rapidly respond following Mtb challenge (27). However, as BCG expresses Ag85b but not ESAT-6, we wanted to determine if BCG vaccination would overcome the impaired T cell migration to the lung parenchyma observed in unvaccinated pMT-10 mice overexpressing IL-10 (14). Our histological analysis showed that T cells from B6 mice infiltrated the granuloma whereas in pMT-10 mice accumulated in perivascular cuffs (Figure 3A). To confirm this observation and determine the location of $CD4^+$ T cells in the lungs of B6 and pMT-10 mice, we performed intravital flow cytometry at day 30 post-infection. To do this, we intravenously injected mice with a fluorochrome-labeled CD45.2 antibody, and sacrificed them 3 minutes later. Lungs were then collected and processed for flow cytometry analysis, as previously described (14). Our results revealed that vaccinated B6 mice had a reduced frequency of intravascular $CD4^+CD44^+$ T cells than control B6 mice (Figure 3B). This finding supports previous studies showing that the presence of parenchymal $CD4^+$ T cells in the lungs of infected mice correlates with control of infection (28, 29). We also found that the frequency of intravascular $CD4^+$ T cells was similar in the lungs of pMT-10 mice irrespective of their vaccination status (Figure 3B). Importantly, pMT-10 mice had a higher frequency of $CD4^+CD44^+$ T cells than vaccinated B6 mice, and even unvaccinated B6 mice (Figure 3B). The same data was found for intravascular ESAT-6-specific IFN-γ-producing $CD4^+$ T cells (Figure 3C). Together, these data show that BCG vaccination does not overcome the IL-10-mediated migration impairment of newly activated $CD4^+$ T cell to the lung parenchyma.

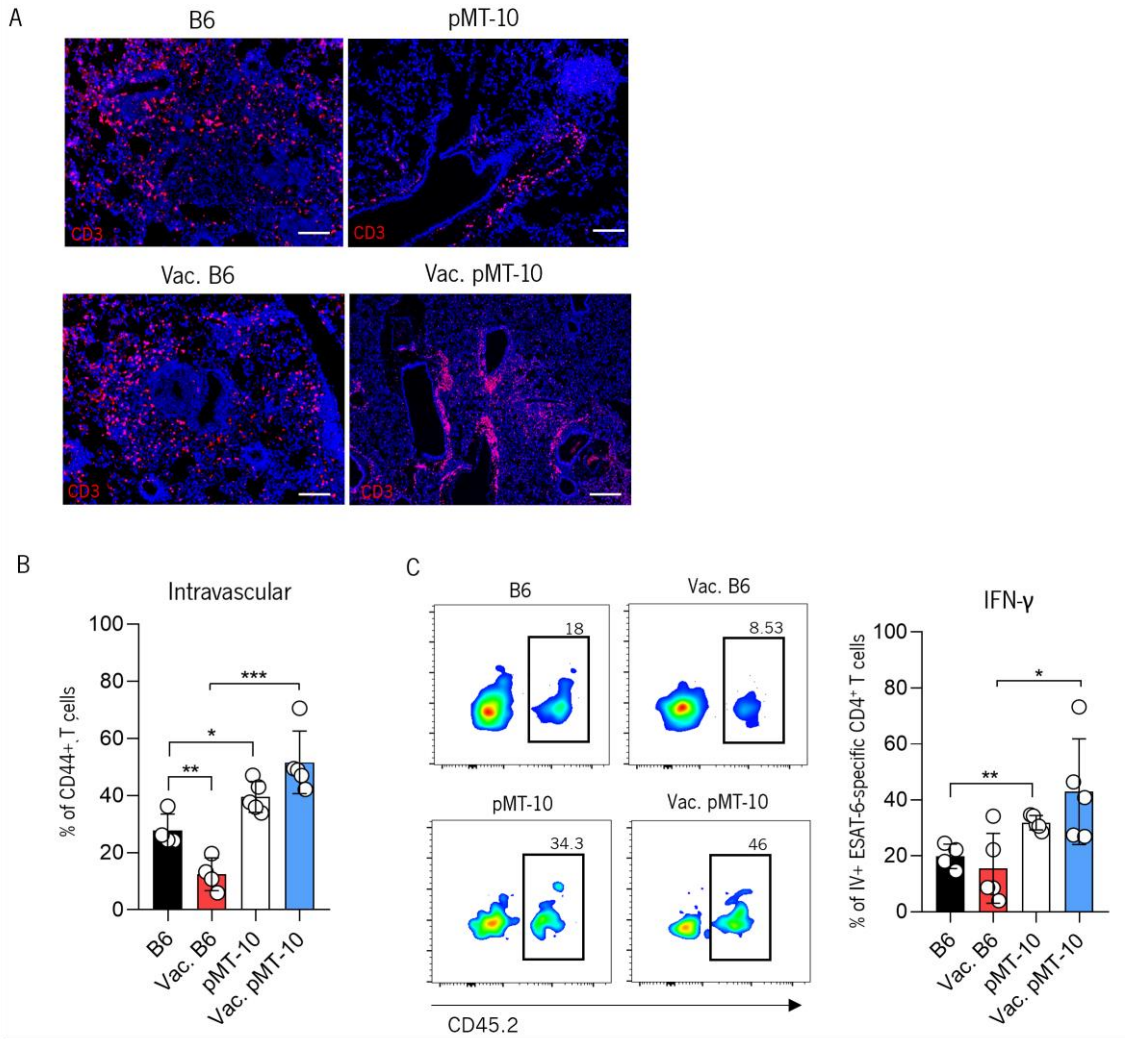

Figure 3- IL-10 expression influences CD4⁺ T cell migration to the lung parenchyma in BCG-vaccinated mice. B6 and pMT-10 mice were vaccinated with1x10⁶ BCG for 60 days prior infection with Mtb H37Rv via aerosol route. IL-10 overexpression was induced 5 days after infection. Unvaccinated mice were used as controls. **(A)** Representative immunofluorescence of CD3⁺ cells in lungs of mice at day 30 after infection. **(B)** Frequency of intravascular (CD45⁺) CD4⁺ T cells in lungs of mice at day 30 post-infection. **(C)** Flow cytometry analysis and frequency of intravascular (CD45⁺) ESAT-6-specific IFN-γ–producing CD4⁺ T cells in lungs of mice at day 30 after infection. Scale bar: 100μm. Data represent 2 independent experiments with 5 mice per group. *p< 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA followed by Tukey's test.

It has previously been shown that the formation of perivascular cuffs in Mtb-infected lungs associates with defective formation of tertiary lymphoid follicles, as determined by the accumulation of B cells within the granuloma (16). Since our data revealed that BCG vaccination did not improve the accumulation of newly activated CD4⁺ T cells in the lung parenchyma of pMT-10 mice, we wanted to determine the impact of BCG vaccination in the formation of tertiary lymphoid follicles. Our histological examination of lung sections of Mtb-infected lungs 30 days after infection revealed a decreased accumulation of B cells (marked with B220) in the granuloma of vaccinated and unvaccinated pMT-10 mice as compared to their B6 counterparts (Figure 4A). Specifically, vaccinated pMT-10 mice show

reduced B cell areas within granulomas than vaccinated B6 mice, suggesting that BCG vaccination does not overcome the IL-10-dependent impaired development of lymphoid follicles during Mtb infection.

To explore whether the impaired development of these structures was associated with reduced expression of homeostatic cytokines, we assessed the expression of CCL19, CCL21, and CXCL13 in the lungs of vaccinated and unvaccinated B6 and pMT-10 mice (Figure 4B). We found no significant differences in the expression of these chemokines across all groups of mice. Interestingly however, we found that vaccinated pMT-10 mice expressed reduced levels of CCR7 and CXCR5 in the lungs when compared to vaccinated B6 mice (Figure 4B). These findings suggest that IL-10 expression during Mtb infection does not impair the expression of homeostatic chemokines, but it reduces the expression of chemokine receptors essential for the development of tertiary lymphoid follicles in the lungs of vaccinated mice.

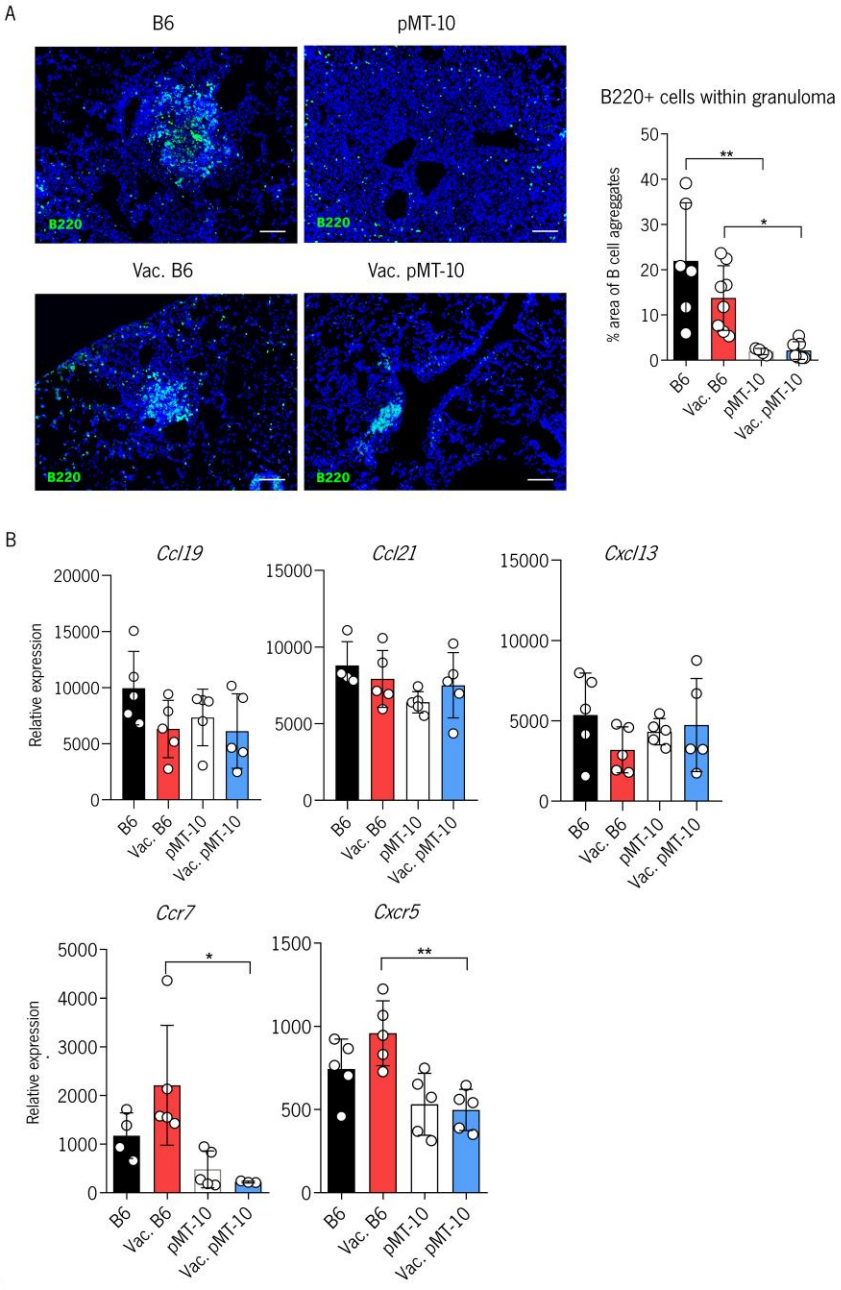

Figure 4- IL-10 expression impacts B cell follicle development in BCG vaccinated mice. B6 and pMT-10 mice were vaccinated with1x10^6 BCG for 60 days prior infection with Mtb H37Rv via aerosol route. IL-10 overexpression was induced 5 days after infection. Unvaccinated mice were used as controls. **(A)** Representative immunofluorescence of B220$^+$ cells in lungs of mice and frequency of granuloma area with B cell aggregates at day 40 after infection. **(B)** Relative expression of chemokine and chemokine receptors. Scale bar: 100µm. Data represent 2 independent experiments with 5 mice per group. *P < 0.05, **P < 0.01 by one-way ANOVA followed by Tukey's test.

In all, our data show that the presence of lung-resident Ag85b-specific T cells prior to Mtb infection is sufficient to overcome the antagonistic effects of IL-10 in the control of Mtb infection. Despite this, BCG vaccination does not appear to overcome the migration deficiency of CD4$^+$ T cells primed in an IL-10 enriched environment, as is the case of ESAT-6-specific T cells, which may have a detrimental impact in the long-lasting control of infection.

4. Discussion

We have recently demonstrated that IL-10 antagonizes the early control of Mtb infection by promoting the development of vasculature-associated $CD4^+$ T cells with impaired ability to migrate to the lung parenchyma (14). This migratory deficit is associated with reduced antigen sensing and ability of $CD4^+$ T cells to engage with infected phagocytes. On the other hand, IL-10 overexpression after the onset of the acquired response does not alter the outcome of chronic Mtb infection (14). These data show that, in the IL-10-enriched environment, $CD4^+$ T cells differentiate into a vasculature-associated phenotype with reduced ability to migrate into the lung parenchyma and infiltrate the infected lesion thereby compromising control of Mtb infection. Therefore, in this work we questioned the impact of IL-10 in the control of Mtb infection in vaccinated hosts. Our data show that IL-10 overexpression during Mtb infection in BCG-vaccinated mice does not interfere with vaccine-induced protection. In both B6 (normal IL-10) and pMT-10 (high IL-10) BCG-vaccinated mice, the control of infection was associated with a rapid accumulation of Ag85b-specific $CD4^+$ T cells in the lungs. On the other hand, there was an impaired migration of ESAT-6-specific $CD4^+$ T cells to the lung parenchyma of mice overexpressing IL-10, as we previously demonstrated (14). As both Mtb and BCG express Ag85b while only Mtb expresses ESAT-6, these data show that IL-10 overexpression does not impair the recall response, but it does impair the primary response. In addition, we found an impaired formation of B cell follicles in the lungs of mice overexpressing IL-10 associated with reduced expression of CXCR5 and CCR7. Taken together, these data suggest that while an IL-10-enriched environment can modulate de novo differentiation and migration of recently activated $CD4^+$ T cells, it does not affect already differentiated T cells.

It has been previously shown that IL-10 regulates BCG-mediated memory development and subsequent control of Mtb infection (30). Herein, we addressed the impact of IL-10 in the protective functions of an already established memory response. Our data showing a reduced impact of IL-10 in the established memory response is in line with our previously published data demonstrating that IL-10 overexpression during the chronic stage of Mtb infection does not impact the outcome of infection (14). These observations together suggest that the impact of IL-10 in disease progression is minimal after the onset of the $CD4^+$ T cell response. Indeed, our previous data showed that the antagonistic role of IL-10 in the control of Mtb infection was mainly in the development of vasculature-associated $CD4^+$ T cells that lack the ability to migrate to the lung parenchyma and engage with infected phagocytes (14). Therefore, these observations taken together with previous data showing that protection conferred by BCG vaccination is dependent on memory lymphocytes retained in the lung (27), suggest that the reduced impact of IL-10 in vaccinated mice is likely because a subset of memory lymphocytes is already colonizing

the lungs. In accordance with these data, we found a similar expansion of Ag85b-specific T cell responses at day 15 post-Mtb infection in both B6 and pMT-10 vaccinated hosts. However, we cannot exclude the possibility that memory T cells generated by BCG vaccination are more resilient to the immunosuppressive effects of IL-10. In this regard, it has been recently demonstrated that mice vaccinated with the second-generation recombinant BCG30 vaccine and treated with the molecule inhibitor of the IL-10/STAT3 axis, 5, 15-diphenyl porphyrin (DPP), after challenge with Mtb aerosol infection, resulted in the increased proliferation of central and effector memory T cells compared with untreated mice (31). These findings suggest that IL-10 signaling is similar in both naïve and memory T cells. As such, our results are best explained by the protective effect of lung-resident $CD4^+$ T cells that rapidly control Mtb infection. Additionally, they further support our previous data showing that the main antagonistic effect of IL-10 is in the development of vasculature-associated $CD4^+$ T cells (14).

Direct interaction between $CD4^+$ T cells and infected phagocytes is essential to control Mtb growth (15). Recent evidence suggests that the migration of T cells into the lung parenchyma, specifically CXCR5-expressing T cells, is associated with the development of ectopic lymphoid structures characterized by the formation of B cell follicles. The formation of these ectopic structures has been suggested to facilitate optimal positioning of T cells promoting the development of a more protective granuloma (16). Indeed, a defective development of ectopic lymphoid structures is associated with poor control of infection both in humans and in experimental animal models of Mtb infection (16, 20, 32). Despite this, the precise mechanisms whereby ectopic lymphoid structures promote better control of infection remain unclear. Our study supports the association between reduced T cell infiltration in the lung parenchyma and the defective formation of ectopic lymphoid structures, suggesting that these cells play a key role in the development of these structures. However, it has been recently demonstrated that group 3 innate lymphoid cells expressing CXCR5 also play a crucial role in the formation of ectopic lymphoid structures and in the control of Mtb infection (33). IL-10 may modulate the activity of these innate lymphocytes to hamper the generation of B cell follicles. While we did not analyze the activity not accumulation of group 3 innate lymphoid cells, we did find reduced expression of CXCR5 and CXCR7 in the lungs of pMT-10 mice. Nevertheless, the fact that BCG vaccination confers early protection in these mice shows that ectopic lymphoid structures are not essential for the early control of infection. However, it is possible that the long-term control of infection may not maintained in the absence of ectopic lymphoid follicles. Future experiments are required to address this question.

BCG vaccination has been shown to confer 60-80% protective efficacy against tuberculous meningitis in children (4); however, its efficacy against adult pulmonary TB is highly variable (34). While this work aimed to mechanistically define the impact of IL-10 overexpression in the T cell response specifically after vaccination, these data may have important clinical implications, specifically in individuals exposed to Mtb that may have co-infection with SARS-CoV-2 (35), influenza (36), or malaria (37) and consequently produce high IL-10. Our data suggest that IL-10 overexpression in these patients should not impair BCG-induced protection; however, if indeed the development of B cell follicles is critical for long-term control of Mtb infection, the overexpression of IL-10 may contribute to the reactivation of Mtb infection. Future research is necessary to clarify this issue.

Overall, our data show that BCG vaccination prevents the antagonistic effects of IL-10 in the early control of Mtb infection. Despite this, the primary $CD4^+$ T cell response generated against antigens not expressed by BCG remains affected by IL-10, resulting in increased $CD4^+$ T cell accumulation in the lung vasculature and reduced formation of B cell follicles.

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Chapter IV

General Discussion and Future Perspectives

1. General Discussion and Future Perspectives

During the past two years, the COVID-19 pandemic caused significant setbacks towards the goal set forth by the WHO of ending tuberculosis (TB) by 2035. The increased TB death rates, together with a 15% reduction in the number of people treated for drug-resistant TB, and the 21% reduction in people receiving preventive treatment are serious predictors of a major global health concern (1). With TB being far from a disease of the past, novel therapeutic and preventive approaches are urgently necessary.

Infection with *M. tuberculosis* (Mtb) elicits a strong cell-mediated immune response. Although the collaboration between the innate and acquired immune response is key for the control of Mtb infection, the fundamental role of $CD4^+$ T cells is undeniable (2–5). Accordingly, BCG vaccination induces a greater accumulation of lung-resident memory IFN-γ-producing $CD4^+$ T cells that associates with improved control of bacterial proliferation (4, 6–8). Based on this premise, vaccines against TB have been developed to target this immune cellular population. However, efficacy trials for new vaccine regimens reveal that promoting the expansion of these cells will not improve protection beyond BCG (9–11). Therefore, further increasing the numbers of memory IFN-γ-producing $CD4^+$ T cells does not correlate with protection.

Memory responses to mycobacterial antigens are characterized by an earlier accumulation of IFN-γ-producing $CD4^+$ T cells in the Mtb infected lungs (8, 12–14). For instance, mice infected with Mtb and treated with antibiotics to clear the infection, once reinfected display an accelerated ESAT-6-specific $CD4^+$ T cell response when compared to naïve mice (15, 16). The same findings are observed for the Ag85b-specific $CD4^+$ T cell response, although at a smaller extent (15). However, this early accumulation occurs about five days earlier than in unvaccinated mice (8, 12, 14). Indeed, in unvaccinated mice infected with Mtb begin controlling infection at day 21 whereas this control begins at day 15 in BCG-vaccinated mice (8, 12, 14). On the other hand, T cell response to infections such as *Listeria monocytogenes*, influenza, or lymphocytic choriomeningitis virus (LCMV) is much faster, occurring as soon as 7-8 days after infection (17). These data show that the delayed onset of the acquired immune response to aerosol Mtb infection, including after vaccination. Importantly, this delay allows Mtb to grow in the lungs and engender a local inflammatory microenvironment that favors its persistence (18–20). Therefore, understanding how to accelerate the expression of the acquired immune responses, particularly IFN-γ-producing $CD4^+$ T cells that rapidly control Mtb infection is key for the development of more efficient vaccine regimens.

The expression of immunosuppressive markers is essential to prevent immunopathology. However, the heterogeneity of these markers causes different implications in Mtb infection control. For instance, it has previously been shown that PD-1-expression is required to prevent $CD4^+$ T cells-dependent immunopathology. In fact, loss of PD-1 expression on $CD4^+$ T cells in Mtb infected mice leads to increased

mortality during the early stages of infection (21). On the other hand, KLRG1 has been demonstrated to induce a terminal differentiation state of $CD4^+$ T cells that is detrimental for the control of infection, contributing for increased mortality (22, 23). Therefore, further understanding the mechanisms employed by the different immunoregulatory molecules might be key to unravel new correlates of protection and of susceptibility. In this regard, the immunosuppressive cytokine IL-10 emerged as a central regulator of the protective T cell-mediated immune response against Mtb and has been shown to have a detrimental impact in the control of Mtb infection (24–26). However, the precise immunological consequences of IL-10 production throughout Mtb infection remain unknown. In this doctoral thesis, I purposed to explore the key mechanisms employed by IL-10 to regulate the protective pulmonary $CD4^+$ T cells and to understand how these contribute for the control of Mtb infection.

In Chapter II, the novel mouse model of controlled IL-10 overexpression recently described (pMT-10) (27) was used to define the temporal role of IL-10 during Mtb infection. Our approach using a mouse model wherein IL-10 overexpression can be controlled overcame the interpretation constraints of previous experiments using animal models that were deficient or constitutively overexpressed IL-10. In this regard, C57BL/6 mice produce low levels IL-10 following Mtb infections when compared to more susceptible mouse strains, such as BALB/c or CBA mice (28, 29). It is therefore not surprising that the outcome of the Mtb infection in IL-10-deficient mice of C57BL/6 background is similar to that of Wild-type C57BL/6 mice (30). On the other hand, transgenic mouse models of conditional IL-10 overexpression have also been used to address the impact of IL-10 in the control of Mtb growth. Specifically, mice overexpressing IL-10 under the control of the IL-2 (31, 32), or CD68 (33) promotors have been shown to display enhanced susceptibility to Mtb infection; this despite the fact that the kinetics of IL-10 expression is challenging to determine thus hindering the analysis of the mechanisms whereby IL-10 impairs control of infection. In this context, the pMT-10 mouse model allowed for the first time the immediate overexpression and cessation of IL-10 in different stages of Mtb infection.

The studies from Chapter II revealed that IL-10 has a differential impact in the control of Mtb proliferation depending on the stage of the infection. Indeed, IL-10 overexpression during the early stages of infection, but not at the late stages of infection, had an important impact in the disease outcome with mice overexpressing IL-10 displaying increased bacterial burdens and exacerbated lung pathology. Crucially, the uncontrolled bacterial proliferation and exacerbated lung pathology were associated with a delayed accumulation of IFN-γ-producing $CD4^+$ T cells in the lungs of 5-7 days comparing with mice with normal levels of IL-10. These findings revealed for the first time that IL-10 plays a key role in delaying the expression of T-cell mediated immunity in the Mtb-infected lungs. However, immune therapies targeting

IL-10 signaling have been described to induce moderate to high severity side effects, in a dose-dependent manner (34–36). Therefore, identifying the molecular pathways whereby IL-10 mediates its effects may be essential for the development of novel therapeutic targets.

1.1. Potential IL-10-induced molecular mediators impairing the onset of the primary immune response

The development of $CD4^+$ T cell responses comprise different stages and occur in distinct organs. Studies using the mouse model of TB demonstrate that upon the deposition of Mtb-infected aerosol droplets in the lower airways, patrolling innate immune cells capture the bacteria and migrate through the lymphatics into the mediastinal lymph nodes (mLN) one week after infection (4). Within the mLN, antigen-presenting cells (APCs) initiate T cell priming. Antigen presentation in the mLN is a limiting step for the time-appropriate onset of T cell immunity and for the control of infection (37, 38). In fact, more resistant mouse strains such as C57BL/6 mice display an earlier accumulation of APCs carrying Mtb peptides within the mLN when comparing with more susceptible mouse strains like C3H/HeJ (17). In accordance with these findings, chapter II discloses a delay in $CD4^+$ T cell priming and differentiation of approximately 2 days in mice overexpressing IL-10. The precise mechanism responsible for this delay is yet to be unveiled. It has been demonstrated that IL-12p40 plays a key role in the migration of DC to the mLN to initiate T-cell priming. In this regard, IL-10 has been shown to suppress IL-12 production, thus preventing DC trafficking to the mLN (39, 40). In our experiments, we found that mice overexpressing IL-10 display decreased expression of CCR7 when compared to mice expressing normal levels of IL-10 at day 25 after infection (unpublished data). Therefore, it is likely that IL-10 is interfering with $CD4^+$ T cell priming in the mLN by downregulating CCR7 expression by antigen-presenting DCs. Additionally, the well described inhibitory role of IL-10 in MHC-II expression by innate immune cells could also delay $CD4^+$ T cell priming and differentiation (41).

While IL-10 overexpression delayed CD4 T cell priming, at day 16 post-infection we found similar levels of IFN-γ upon restimulation of mLN single cell suspension. These data suggest that in both B6 and pMT-10 mice $CD4^+$ T cell differentiation was similar at this time point. These data suggest that the slight delay in $CD4^+$ T cell priming could not be responsible for the delayed accumulation of $CD4^+$ T cells in the lungs found in pMT-10 mice. Instead, we unraveled a significant impairment in the recruitment of $CD4^+$ T cells from the lung vasculature into the parenchyma soon after the onset of the acquired immune response. These data show that IL-10 not only impacts the initiation of acquired immune response in the mLN, but it also interferes with T cell migration into the lung parenchyma. Importantly, our adoptive transfer experiment show that the IL-10 effects are intrinsic to the $CD4^+$ T cell. Indeed, $CD4^+$ T cells

differentiated in the IL-10 enriched environment maintain their deficient parenchyma migration even in normal IL-10 environments. The presence of Ag-specific CD4+ T cells within the lung parenchyma is essential to allow their direct interaction with infected phagocytes in the nascent granuloma, a process essential for the containment of bacterial expansion (19). In fact, we observed reduced expression of CD69 by CD4+ T cells soon after their arrival from the mLN into the lungs, revealing impaired antigen sensing in the lungs. Consequently, CD4+ T cells also presented impaired abilities to proliferate, thus explaining the reduced frequencies and numbers of these cells early during infection.

The development of different subsets of CD4+ T cells with distinct abilities to penetrate the granuloma has been previously described. In this regard, the chemokine receptor CXCR3 has been recently shown to play a key role in the migration of CD4+ T cells into the lung parenchyma (42). Accordingly, the data from chapter II disclosed that CD4+ T cells developed in an IL-10-rich environment displayed reduced expression of CXCR3. These data together with the adoptive transfer data wherein CD4+ T cells differentiated in an IL-10-rich environment and adoptively transferred into a host with normal levels of IL-10 maintain their phenotype show that IL-10 acts intrinsically on CD4+ T cells to hamper their parenchyma migratory capacity. However, the subset of Mtb-specific effector CD4+ T cells with the best lung-homing capacity seems to co-express CXCR3 with other receptors including CXCR6, CCR2, and CCR5 (42). Therefore, it is unlikely that a single molecular target provides the best alternative to deliver the ideal immune response against Mtb.

The different subsets of CD4+ T cells with distinct abilities to penetrate the parenchyma have also been categorized based on the expression of PD-1 and KLRG1 (18, 43, 44). PD-1-expressing CD4+ T cells are essential for the control of Mtb growth, as they locate within the lung parenchyma and express CXCR3, whereas KLRG1-expressing CD4+ T cells are cuffed around the lung vasculature and are detrimental for the control of Mtb expansion (18, 43, 44). The mechanisms underlying the development of these opposing subsets of CD4+ T cells and the impact of IL-10 expression in their development remains unclear. However, previous findings from our group revealed that mice deficient for IL-27R, a potent inducer of IL-10 production, showed a reduced presence of KLRG1-expressing CD4+ T cell subset in the lungs 60 days post-infection (18). These data show that IL-27 also impacts T cell localization in the lungs, which ultimately impacts the degree of T cell differentiation and expression of the immunoregulatory markers PD-1 and KLRG1. Despite this, in the mouse model of Mtb infection used in this doctoral thesis, no alterations were found in KLRG1 or PD-1 expression until day 40 post-infection (data not shown). Since these mice succumb to infection before day 60, it is difficult to assess whether IL-10 overexpression would induce the accumulation of KLRG1-expressing CD4+ T cells. Nevertheless, the experiments from

Chapter II and III clearly show a causality connection between IL-10 expression and the development of $CD4^+$ T cells with reduced lung-homing capacities.

Altogether, the mouse model used in this thesis can be useful for future assessments on the genes and pathways influencing the expression of a rapid protective T cell response against Mtb infection. The subsequent validation of this signature could provide valuable information regarding correlates of protection and susceptibility against Mtb infection.

1.2. Effects of IL-10 expression during BCG vaccination: Finding hints from the development of the primary immune response

The relevance of lung parenchyma-resident $CD4^+$ T cells during Mtb infection has been also demonstrated in studies using BCG vaccinated mice (45). Indeed, BCG induces the generation of a memory $CD4^+$ T cell population resident in the lung parenchyma that persists for over 12 months after vaccination (7). Interestingly, protective immunity provided by BCG immunization in mice has also been demonstrated to last for up to a year (46, 47). Therefore, it is reasonable to assume that BCG-mediated protection is also linked with the accumulation of a parenchyma-resident memory $CD4^+$ T cells. However, the protection conferred by BCG vaccination is highly variable (48–50). In this regard, mounting evidence suggest that IL-10 expression during memory development might be a critical contributor. Indeed, previous studies have demonstrated that IL-10 inhibits the development of memory T cells in viral infections (51, 52). During mycobacterial infections, blocking IL-10 signaling during BCG vaccination leads to heightened T cell responses and improved control of Mtb (53). In humans, there are also evidences that IL-10 reduces BCG efficiency. Human cord blood cells increase the expression of IL-10 and IL-5 in response to BCG, which likely explains improved T cell responses found in infants vaccinated at ten weeks of age when comparing with newborns (54, 55). Whether the mechanisms whereby IL-10 regulates memory development overlap with the IL-10-mediated regulation of primary effector T cell responses demonstrated in this thesis remains unknown. However, these data highlight the relevance of further exploring the molecular players contributing for reduced generation of BCG-induced lung-resident $CD4^+$ T that could become targets towards improving BCG-mediated protection.

1.3. Effects of IL-10 expression in BCG-induced protective immunity

The data presented in chapter III demonstrate that IL-10 overexpression does not restrain the BCG-mediated early accumulation of Ag85-specific $CD4^+$ T cells. However, IL-10 overexpression did prevent the migration of $CD4^+$ T cells specific to the Mtb antigen ESAT-6 to the lung parenchyma, similarly to what was observed in chapter II for unvaccinated mice. Despite this, BCG vaccination prevented the early

disease progression observed in unvaccinated mice. These findings are in accordance with previous studies demonstrating that mice vaccinated with BCG and subsequently treated with fingolimod, to prevent the egress of recently activated T cells in the mLN, retain the ability to control Mtb (13). However, the data presented in this thesis addresses an Mtb infection rather than a secondary BCG challenge. Since Mtb expresses immunodominant antigens not expressed by BCG, it is possible that the generation of an impaired ESAT-6-specific $CD4^+$ T cell response will have implications in disease outcome at later stages. Additionally, our data does not exclude the potential impact of IL-10 in memory T cells. Indeed, it has been shown that vaccinated mice treated with the molecule inhibitor of the IL-10/STAT3 axis, 5, 15-diphenyl porphyrin (DPP), followed by Mtb aerosol infection, presented increased proliferation of central and effector memory T cells compared with untreated mice (56). Therefore, we do not discard that IL-10 signaling might be influencing somehow the established memory T cell response. Therefore, further understanding how BCG vaccinated mice control chronic Mtb infection under IL-10 overexpression is essential.

The migration of $CD4^+$ T cells into the lung parenchyma has been linked with the development of ectopic lymphoid follicles characterized by the formation of B cell aggregates within granulomas (12, 57–59). For reasons not yet understood, the formation of these tertiary lymphoid structures is critical for the proper interaction of T cells with infected myeloid cells and has been linked with protection during TB disease in humans (57, 60). Interestingly, the development of these lymphoid structures has also been shown to be protective in cancer (61). In this regard, different strategies have been studied to induce ectopic lymphoid structures and the majority of them include modulating the chemokine milieu (61). CCL19, CCL21, and CXCL13 are homeostatic chemokines critical for the formation of ectopic lymphoid structures in the lungs. The reduced expression of CCR7 in the lungs of vaccinated and unvaccinated pMT-10 mice suggest a deficient CCL19 and CCL21 signaling, hence the reduced follicular B-cell aggregation within lymphoid structures. Despite this, previous studies revealed that although mice deficient for CCR7 did not develop organized ectopic lymphoid structures, they were fully capable of controlling pulmonary Mtb growth (62). Nevertheless, one cannot exclude the possibility that CXCL13 production in the lungs of these mice could ensure, at least in part, the efficient migration of T cells to the infected lesions. Indeed, mice deficient for CCL19, CCL21 and CXCL13 are more susceptible when comparing with single knock-out mice, revealing a cooperation of these chemokines in maintaining protective immune responses against Mtb (57, 63). The data presented on chapter III also reveal a decreased expression of CXCR5 in the lungs, which could indicate that the optimal migration of T cells into the lesion is indeed compromised. Therefore, future data aimed at evaluating whether vaccinated

mice pMT-10 mice can maintain control chronic Mtb infection over the long-term may also provide important insights into the role of ectopic lymphoid lesions in predicting TB disease progression.

Collectively, the results presented in this doctoral thesis shed light on the mechanistic pathways and molecules influencing the development of the protective immune response against Mtb infection. Improving current knowledge on the factors that correlate with protection against TB is key for the discovery of prognostic biomarkers and the generation of more efficient therapeutic and preventive strategies.

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