Universidade do Minho
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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Professora Doutora Margarida Correia Neves
do
Professor Doutor Samuel Behar
e do
Professor Doutor Christophe Benoist

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DECLARAÇÃO

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Abstract

Despite significant advances in drug discovery and vaccination strategies during the last decades, infectious diseases are still a major health problem worldwide. Among these, infections by mycobacteria are particularly deadly, and *Mycobacterium tuberculosis* alone – the causative agent of tuberculosis – is estimated to infect a third of the world population and is responsible for 9 million new cases and 1.5 million deaths every year. Interestingly, most people do not develop active disease following infection, but instead are capable of controlling infection and remain in an asymptomatic state termed latent infection. Although how the immune system controls infection is unclear, it is clear that T cells are a key component of a successful response against mycobacteria, since infection with human immunodeficiency virus (HIV) greatly increases susceptibility to infection. Because T cells play such an important role in controlling mycobacterial infections, there is considerable interest in understanding which subsets are necessary for protection, which mechanisms are employed by these cells to limit bacterial growth, and whether such cells can be elicited by vaccination strategies. To clarify the role of T cells during immunity to tuberculosis, we addressed two aspects of T cell biology: how infection impacts the thymus, the organ where T cells differentiate; and how $CD8^+$ T cells mediate protection during tuberculosis.

Our lab had previously shown that mycobacteria could disseminate to the thymus and interfere with thymic function. However, it was unclear whether the immune system could control thymic infection and halt bacterial growth in this organ. Here we show that an immune response is recruited to the infected thymus and controls infection. This response involves the production of pro-inflammatory cytokines by antigen-specific $CD4^+$ and $CD8^+$ T cells, which stimulate infected cells and leads to control of bacterial replication. Interestingly, these T cells are not cells undergoing differentiation in the thymus, but rather are activated cells that recirculate from infected peripheral organs to the thymus to fight infection. This recruitment is associated with the production of the chemokines CXCL9 and CXCL10 in the infected thymus, and recirculating antigen-specific T cells that express CXCR3 and preferentially traffic to the thymus.

In a second set of studies, we investigate how $CD8^+$ T cells contribute to immunity against *M. tuberculosis* infection. Although $CD8^+$ T cells are essential for optimal immunity during tuberculosis, how they exert protection is unclear. Here we describe the generation of a new mouse model, the first carrying T cells expressing a

recombinant T cell receptor (TCR) specific for a *M. tuberculosis* antigen recognized by $CD8^+$ T cells – TB10. By combining single cell sorting of antigen-specific cells with PCR amplification of their TCRs, we selected 4 TCR candidates specific for TB10. These TCRs were cloned into retroviral vectors and used in retrovirus-mediated stem cell gene transfer to generate retrogenic mice. These mice express $CD8^+$ T cells that recognize and respond to their cognate peptide. More importantly, adoptive transfer of retrogenic cells provides protection against aerosol infection with *M. tuberculosis*. We then use these mice to investigate the mechanism by which $CD8^+$ T cells mediate protection. By using retrogenic mice that are deficient in the production of interferon-γ or perforin, we show that protection mediated by $CD8^+$ T cells requires cytokine production but is independent of their cytotoxic capacity.

Collectively, the studies presented in this thesis clarify different aspects of T cell-mediated immunity to mycobacteria; they reinforce that the thymus is not an immune privileged organ and should be studied in the context of infection, and strengthen the notion that $CD8^+$ T cells are protective after *M. tuberculosis* infection.

Resumo

Nas últimas décadas observamos incríveis avanços tanto no desenvolvimento de vacinas como de antibióticos. No entanto, as doenças infecciosas continuam a ser um problema de saúde global. Entre estas, as infecções por micobactérias são particularmente mortiferas, e é estimado que um terço da população mundial esteja infectada com *Mycobacterium tuberculosis* – a bacteria responsável pela tuberculose. Esta doença causa 9 milhões de novos casos todos os anos, resultando em cerca de 1.5 milhões de mortes anuais. No entanto, a maioria dos indivíduos infectados não desenvolve a doença, e permanence num estadio clinico assimptomatico designado por infecção latente. Apesar de ainda não ser claro como é que o sistema imunitário controla estas infecções, as células T são um componente essencial neste processo, já que infecção com o virus de imunodeficiencia humana (VIH) aumenta significativamente a susceptibilidade à infecção com micobactérias. Devido à importância das células T nestes casos, é importante clarificar que subconjuntos destas células são necessários para controlar a infecção, como é que essas células são protectoras, e se é possível gerá-las por intermédio de vacinas. Para melhor entender qual o papel das células T durante infecções por micobactérias, focamo-nos em dois pontos: qual o impacto destas infecções no timo, o orgão em que as células T se diferienciam; e qual o papel das células T $CD8^+$ durante a tuberculose.

Quando iniciamos estes estudos, era sabido que micobactérias são capazes de disseminar para o timo e interferir com a função deste orgão. No entanto não era claro se o timo consegue controlar estas infecções, e como o faz. Nos estudos aqui apresentados demonstramos que o timo controla a infecção, num processo dependente do recrutamento de uma resposta imunitária adquirida para este orgão. Esta resposta envolve a produção de citocinas pró-inflamatórias por parte de células T, tanto $CD4^+$ como $CD8^+$. Estas citocinas activam as células infectadas, resultando no controlo do crescimento bacteriano no timo. Apesar do timo ser o orgão em que as células T se diferenciam, os nossos estudos revelam que as células responsáveis pelo controlo bacteriano neste orgão não são células que se encontram neste orgão no processo de diferenciação, mas antes células T activadas que recirculam dos orgãos periféricos para o timo para controlar a infecção. Este processo depende da produção das quimoquinas CXCL9 e CXCL10 no timo infectado, e da expressão do receptor CXCR3 por parte das células que recirculam.

Num conjunto de estudos diferentes debruçamo-nos sobre a contribuição das células T $CD8^+$ durante a tuberculose. Apesar de ser aceite que estas células são importantes durante a infecção com *M. tuberculosis*, o mecanismo pelo qual elas protegem indivíduos infectados é ainda desconhecido. Para investigar esta questão criamos um novo modelo animal, o primeiro ratinho que expressa células T $CD8^+$ específicas para um antigénio de *M. tuberculosis*, o antigénio TB10. Para tal, células específicas para TB10 foram isoladas individualmente por citometria de fluxo, os seus receptores de células T (TCR) amplificados por reacção em cadeia de polimerase (PCR) e sequenciados. Das sequências detectadas, 4 foram seleccionadas e clonadas em vectores retrovirais, que foram depois usados para gerar animais retrogénicos, via transferência adoptiva de células estaminais infectadas com os retrovirus. Estes animais expressam células T $CD8^+$ que reconhecem o antigénio de interesse, e transferência adoptiva de células retrogénicas é suficiente para proteger significativamente animais de infecção aerogénica com *M. tuberculosis*. Para investigar qual o mecanismo responsável pela protecção observada, geramos animais retrogénicos incapazes de produzir interferão-γ ou perforina, e demonstramos que as células T $CD8^+$ precisam de produzir citocinas, mas não de ser citotóxicas, para proteger animais contra infecção por tuberculose.

Em conjunto os estudos apresentados nesta tese clarificam diferentes aspectos acerca da imunidade mediada por células T durante infecções com micobactérias: re-enforçam a ideia de que o timo não é um orgão imuno-priveligiado e deve ser estudado no contexto de doenças infecciosas; e que as células T $CD8^+$ são protectoras no contexto de tuberculose.

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Introductory note

The work reported in this dissertation focus on two distinct, yet related projects, which I will try to present in an organized fashion. I will start with a brief introduction on the functions of the immune system, the role of the thymus and T cells in immunity, and how these participate in the overall immune response to tuberculosis. This will be **chapter 1**. After this, the thesis will be divided on 2 main sections.

The first section compiles a set of studies that focus on the consequences of thymic infection by mycobacteria and its impact on ongoing immune responses. Historically, this was the original focus of my studies when I started my PhD in Braga, Portugal, and a big part of my initial studies after I moved to Boston. This section starts with a review (currently under revision) about the problem of thymic infection (**chapter 2**), followed by a manuscripts published in *The Journal of Immunology* in which I am a co-first author (**chapter 3**). This is a report entitled *"T cells home to the thymus and control infection"*, where we show that T cells re-circulate from the periphery to the thymus to control mycobacterial infection.

The second section focus on the role of $CD8^+$ T cells during *Mycobacterium tuberculosis* infection and our efforts to develop an animal model to improve our understanding of what constitutes protective immunity to tuberculosis. This has been the subject of my work for most of the time since I moved to Boston. This section starts with a brief review on the role of $CD8^+$ T cells during *M. tuberculosis* infection (**chapter 4**). This introduction serves as the rationale to the development of a new animal model to study $CD8^+$ T cell responses following *M. tuberculosis* infection. In **chapter 5** we describe the use of retrogenic technology to generate a new mouse model expressing a large frequency of T cells specific for the immunodominant TB10 antigen. This chapter also includes the data generated so far by using this new mouse model, presenting how we used retrogenic mice to study $CD8^+$ T cell priming following infection and the mechanisms responsible for $CD8^+$ T cell protection during tuberculosis.

Finally, **chapter 6** includes a general discussion that focus on the causes and consequences of thymic infection, particularly in settings of chronic infection; on the role of $CD8^+$ T cells in the mouse model of tuberculosis and how retrogenic mice can be used in this context; and on the relationship between the two projects, with an overall model on the role of T cells during tuberculosis, both in the thymus and the periphery.

This thesis revolves around the study of immunity to tuberculosis, the disease caused by infection with the pathogen *Mycobacterium tuberculosis.* Because these studies relate to different aspects of immunity, I decided to divide them into two sections. The first focuses on the role of the thymus during mycobacterial infection; the second on the study of $CD8^+$ T cell responses during tuberculosis. The current literature related to each topic is reviewed at the start of each section. In this chapter, I will try to summarize the state-of-the-art of what is common between both projects, focusing on how T cells are generated and how they participate in the immune response against mycobacteria

Chapter 1

Introduction

T cell immunity to tuberculosis: the role of the thymus in T cell generation and how T cells contribute to host defense against tuberculosis

Introduction

immunity (noun): the quality or state of being immune; *especially*: a condition of being able to resist a particular disease especially through preventing development of a pathogenic microorganism or by counteracting the effects of its products (*Merriam-Webster dictionary*).

We live surrounded by microorganisms, yet do not get sick from them all the time. This is because, over a period of several hundred million years, multicellular organisms have developed a dedicated system to prevent, detect and fight infection[1]. This **immune system** consists of multiple strategies to protect the body from infectious agents and their consequences, and involves several effector cell types and different immune molecules. These strategies are commonly divided in two major branches, referred to as innate versus adaptive immunity. The major differences between these two systems are kinetics, specificity and the ability to generate immunological memory[2].

Innate immunity usually refers to components that mount a fast response against infectious agents in a non-specific way, creating a front line of defense against pathogens. These responses are usually dependent on the recognition of general features of infectious agents, by detection of either pathogen or danger associated molecular patterns (PAMPs or DAMPs) by specific receptors on immune cells – toll-like receptors (TLRs) or pattern recognition receptors (PRRs)[3,4]. Because the innate immune system recognizes general features of microbes, it is not capable of immunological memory, i.e., the innate immune response to a subsequent challenge with the same pathogen is usually not faster or stronger than the first encounter. Important components of innate immunity are dendritic cells, monocytes and macrophages, granulocytes (neutrophils, basophils, eosinophils and mast cells), the complement system, and natural killer (NK) cells.

In contrast, **adaptive immunity** comprises a specialized set of cells with the capacity to individually recognize each invading pathogen and mount specific immune responses. The major constituents of adaptive immune responses are B and T cells. B cells are responsible for antibody mediated immune responses (or humoral immunity), and T cells for cell mediated immunity. These cells are characterized by the surface expression of specific receptors that are the result of random gene rearrangements, therefore yielding virtually endless combinations that are capable of

detecting essentially every antigen present in bacteria, fungi, virus and parasites. This specificity allows the adaptive immune system to respond faster and stronger to a secondary challenge with the same pathogen, a feature described as immunological memory[2].

Despite the division of immune responses in these two branches of innate and adaptive immunity, successful clearance of infectious agents requires a balanced interplay of several components of the immune system.

T cells and the thymus

One of the key components of the adaptive immune system are T cells, which are defined by the surface expression of the T cell receptor (TCR). There are several T cell subsets, whose TCR recognizes different molecules. Conventional T cells recognize small peptide fragments bound to major histocompatibility complex (MHC) molecules expressed on the cell surface[5]. NKT cells recognize lipid antigens in the context of CD1 molecules. For other subsets, such as γδ T cells and MAIT cells, it is still unclear what molecules they recognize. Nonetheless, each T cell expresses a TCR, a heterodimer consisting of a pairing of an alpha (α) chain with a beta (β) chain – in the case of αβ T cells – or a gamma (γ) chain with a delta (δ) chain – in the case of γδ T cells *(for simplicity reasons we will focus on conventional T cells in this introduction, as they are the main scope of this thesis*).

The T in T cells stands for **T**hymus, the organ where these cells differentiate. T cell precursors are generated in the bone marrow and become functional after differentiation within the thymus. The deceptively simple anatomical structure of the thymus belies its sophisticated ability to generate self-restricted and mostly self-tolerant naïve T cells expressing a broad TCR repertoire capable of recognizing virtually any foreign antigen (and some self antigens). This complex task is achieved by a process that involves somatic recombination of TCR gene segments to generate diversity coupled with a succession of interactions between differentiating thymocytes and resident cell populations that mediate positive and negative selection (reviewed in extensive detail in *Starr et al.*[6]). Briefly, when common lymphoid progenitors (CLPs) arrive in the thymus from the bone marrow, they are called double negative (DN) T cell precursors, due to the absence of the CD4 and the CD8 co-receptors at the surface of these cells[7]. Upon entering the thymus, T cell precursors start to express recombination-activating gene 1 (Rag-1) and Rag-2

genes, which are essential for TCR rearrangement[8]. Cells that successfully rearrange and express a TCRβ chain and pair it with the pre TCRα (pTα) at the cell surface pass beta-selection[9]. After this stage, these cells proliferate rapidly, rearrange the TCRα chain, and start expressing both CD4 and CD8 co-receptors, earning the designation of double positive (DP) cells[7]. DP cells can now undergo selection, in a process that preserves naïve T cells that can recognize peptides presented by MHC (self-restricted) and eliminates most T cells that recognize self-peptides (self-reactive). Selection is dependent on the affinity of the interaction between the TCR expressed by the T cell with the self-peptide-MHC complexes expressed at the surface of thymic resident cells. In this way, DP thymocytes expressing TCRs that are capable of recognizing these complexes with sufficient affinity/avidity receive a positive surviving signal, while cells whose TCR is incapable of such recognition die by neglect – this is called positive selection, and ensures that T cells are self-restricted[10]. Upon positive selection, developing thymocytes undergo yet another selection event. In this step, thymocytes carrying a TCR with high affinity/avidity to the self-peptide-MHC complexes are instructed to die by apoptosis – this is called negative selection, and ensures that most self-reactive T cells do not leave the thymus, preventing autoimmune responses in the peripheral tissues[11]. Of note, some self-reactive T cells escape thymic selection and make it to the periphery, while other cells with high affinity/avidity to self-peptides develop into regulatory T cells (T_{reg})[12]. It is also during thymic selection that DP cells become either CD4 or CD8 single positive (SP) thymocytes. This is determined by what MHC molecules their TCR recognizes, and $CD4^+$ T cells are restricted to class II MHC (MHC-II) while $CD8^+$ T cells are restricted to class I MHC (MHC-I)[6]. After thymic selection, naïve T cells exit the thymus and migrate to peripheral lymphoid tissues, where they reside until they become activated and participate in immune responses (Figure 1).

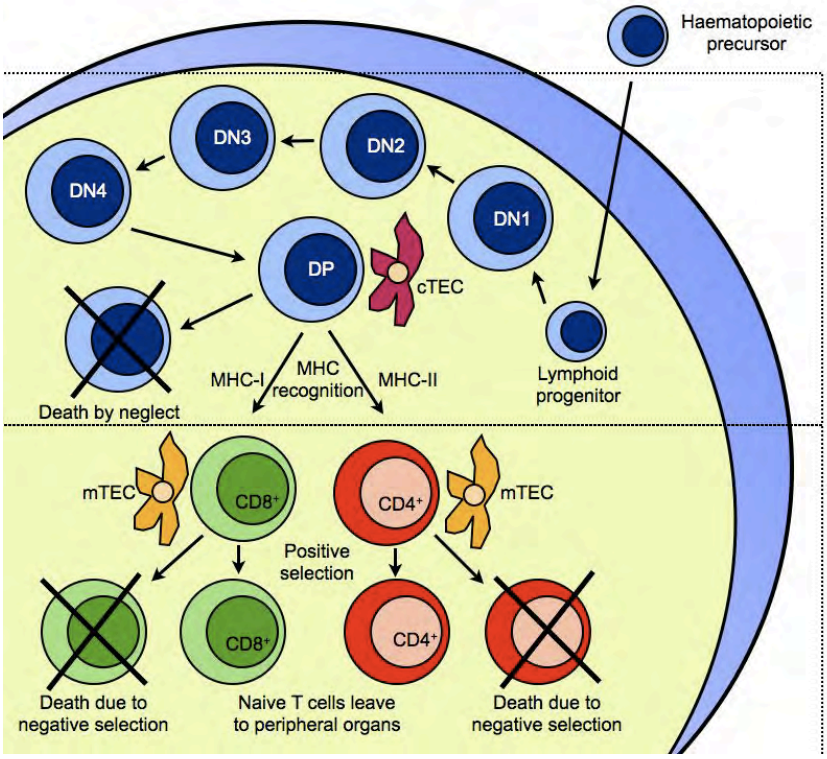

Figure 1. T cell selection in the thymus. T cell precursors enter the thymus, where they undergo a series of events before differentiating into naïve $CD4^+$ or $CD8^+$ T cells. Such events include both positive and negative selection, ensuring that T cells are self-restricted, and for the most part, self-tolerant. DN – double negative; DP – double positive; MHC – major histocompatibility complex; cTEC – cortical thymic epithelial cell; mTEC – medullary thymic epithelial cell.

$CD4^+$ and $CD8^+$ T cells

Despite all the similarities in their development, $CD4^+$ and $CD8^+$ T cells are thought to play different roles in immunity. $CD4^+$ T cells, or helper T cells (T_h), recognize peptides presented by MHC-II, which is only expressed in antigen presenting cells (APC). These peptides are usually derived from within vesicles resulting from the phagocytosis of infectious agents, and therefore $CD4^+$ T cell responses are characterized by the production of cytokines that act on the presenting cell as well as the neighboring cells – hence the term helper cell. Interestingly, naïve $CD4^+$ T cells (T_h0) differentiate into very different subsets depending on the context of the activation and the transcription factors they up-regulate (reviewed by *Zhou et al.*[13]). Briefly, T_h1 development is regulated by interleukin-12 (IL-12) and the T_h1-specific T box transcription factor (T-bet). These cells are characterized by the

production of interferon-γ (IFNγ) and promote protective immunity against intracellular pathogens. T_h2 cells promote humoral immunity and are involved in responses against extracellular parasites. They are also important in the context of allergic reactions and asthma. Their development is regulated by the transcription factor GATA-3, and they produce cytokines such as IL-4, IL-5 and IL-13. T_h17 cells produce IL-17, and are important in the response against extracellular bacteria and fungi. Their inflammatory capacity can also promote autoimmunity. T_{reg} are immunosuppressive cells that express the forkhead box P3 (FoxP3) transcription factor and produce IL-10 and transforming growth factor β (TGF-β). Finally, follicular helper T cells (T_{FH}) promote germinal center responses through secretion of IL-21 and their development is dependent on the B-cell lymphoma 6 (Bcl-6) transcription factor (Figure 2).

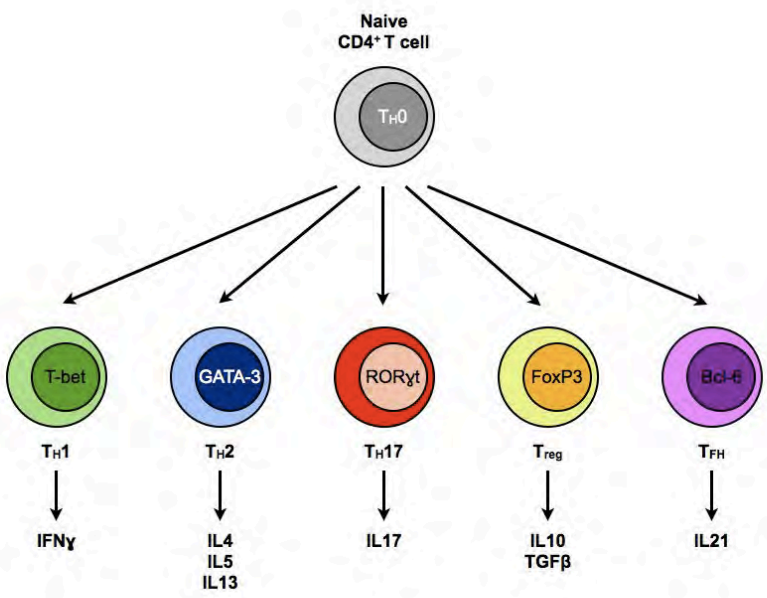

Figure 2. $CD4^+$ T cell subsets. Naïve $CD4^+$ T cells differentiate into several distinct subsets that modulate different functions during immune responses. Differentiation of each subset is associated with the specific transcription factor indicated in the figure and each population is characterized by the production of the indicated signature cytokines.

On the other hand, $CD8^+$ T cells, also called cytotoxic T cells (CTL or T_c), recognize peptides presented by MHC-I, which is expressed by almost every nucleated cell in the organism. These peptides are usually from cytoplasmic origin, and therefore $CD8^+$ T cell responses are important in the fight against viruses,

tumors and intracellular pathogens. $CD8^+$ T cells can directly kill the target cell, and CTL activity involves the secretion of cytotoxic granules, such as granzymes and perforin (and granulysin in humans). In addition to cytotoxicity, cytokine production by $CD8^+$ T cells, such as IFNγ and tumor necrosis factor (TNF), also contributes to host defense[14].

Despite the differences between $CD4^+$ T cells and $CD8^+$ T cells, in terms of both the cells they recognize and their effector mechanisms, these two subsets collaborate in response to immunological challenge and their interaction is key to control infection.

Tuberculosis

tuberculosis (noun): a usually chronic highly variable disease that is caused by a bacterium of the genus Mycobacterium (*M. tuberculosis*) and rarely in the United States by a related mycobacterium (*M. bovis*), is usually communicated by inhalation of the airborne causative agent, affects especially the lungs but may spread to other areas (as the kidney or spinal column) from local lesions or by way of the lymph or blood vessels, and is characterized by fever, cough, difficulty in breathing, inflammatory infiltrations, formation of tubercles, caseation, pleural effusion, and fibrosis – called also TB (*Merriam-Webster dictionary*).

One of the central purposes of the immune system is to protect the body from infectious agents, in what can be seen as a battle between immune cells and the invading pathogens. These battles are some times fast and intense, and usually result in either death of the host or rapid clearance of the infection – such is the case for multiple bacterial and viral **acute infections**. Other times, the battle is long, and does not always lead to sterilization – we call these **chronic infections**. One such example, and arguably one of the most interesting examples of host-pathogen interactions is tuberculosis, the disease caused by *Mycobacterium tuberculosis*.

Mycobacterial infections are among the major health threats worldwide, and humans all over the world are frequently exposed to bacteria from the *Mycobacterium* genus[15]. This genus includes not only *M. tuberculosis* - responsible for tuberculosis, the leading cause of death from a curable infectious disease in the world – but also several other pathogens such as *Mycobacterium bovis*, *Mycobacterium leprae*, *Mycobacterium ulcerans* and *Mycobacterium avium*[15]. *M. tuberculosis* alone is thought to infect around one third of the world's population, and causes an estimated 1.7 million deaths every year[16]. The enormous difference between the number of infected people and the amount of annual deaths is explained by the observation that the majority of infected individuals do not develop active disease but instead keep infection under control in a state termed **latent infection.** In fact, only a small fraction of the infected population, around 5-10%, ever display active disease and is able to transmit disease, for reasons still unclear[17]. However, in the last few decades, and despite potentially curative drugs to control tuberculosis, mycobacterial infections have gained a renewed significance. This is mainly due to two reasons: the spread of human immunodeficiency virus (HIV) infection, which significantly increases the progression to active disease[18]; and the

emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis*[19].

There is currently only one vaccine available to prevent mycobacterial infections, although multiple ventures are currently trying to expand this panel. The licensed vaccine is a live attenuated form of *M. bovis, M. bovis*-bacillus Calmette-Guérin (BCG) that has been used for over 90 years to prevent tuberculosis. Although capable of protecting infants from some severe forms of the disease – such as miliary tuberculosis – vaccination with BCG displays highly variable efficiency in preventing adults from pulmonary tuberculosis, and the mechanism underlying the loss of BCG-induced protection in adults is still not clear[20,21]. This is an important example of the shortcomings of our fundamental knowledge of what constitutes protective immunity to tuberculosis. This lack of information hinders our ability to prevent, fight and eradicate this disease.

Immune responses to mycobacteria

We are still a long way from fully understanding the delicate interplay between the immune system and mycobacteria, but that is not to stay that significant progress has not been made. Over the last decades, several aspects of the immune response against mycobacteria have been addressed, mostly focusing on the initial recognition of bacterial infection by cellular components of the innate immune system and on the role of T cell immunity in controlling the infection.

M. tuberculosis is a facultative intracellular pathogen, usually transmitted by the aerosol route, when an infected individual coughs up droplets containing the infectious agent[22]. Once inhaled by a new host, *M. tuberculosis* is able to invade host cells, and is thought to be initially taken up by alveolar macrophages[23]. In addition to tissue resident macrophages, other phagocytes can harbor *M. tuberculosis* in the lung, such as dendritic cells (DC)[24], monocyte-derived macrophages[25] and neutrophils[25,26]. In addition, *M. tuberculosis* can be found within non-phagocytic cells, such as epithelial cells[27] and adipocytes[28]. How exactly *M. tuberculosis* is taken up by phagocytes is still unclear, but several receptors have been described to participate in this process, such as complement receptors, scavenger receptors and C-type lectin receptors[17]. Interestingly, once taken up by cells, *M. tuberculosis* is able to modulate host immunity, resist killing, and survive and even divide within the infected cell. Part of the reason why *M. tuberculosis* is able to evade host immunity is

its production of specific virulence factors that prevent lysosome fusion to the phagosome, which allows it to evade lysosomal degradation[29-31]. Additionally, *M. tuberculosis* is able to escape from the phagosome to the cytosol[32]. Another way by which *M. tuberculosis* interferes with host immunity is by affecting the cell death modality of the infected macrophage (reviewed by *Behar et al.*[33]). We and others have shown that *M. tuberculosis* modulates the production of lipid mediators within the macrophage, stimulating infected cells to die by necrosis – which promotes bacterial spreading to neighboring cells – and inhibiting apoptosis[34,35]. The inhibition of apoptotic cell death of the infected macrophage impacts negatively on the outcome of the infection, since we have also demonstrated that apoptosis promotes bacterial control, at least partially by the means of efferocytosis[36].

On the other hand, the immune system possesses several ways to stimulate the intracellular killing of *M. tuberculosis* within macrophages, which include the production of the cytokines IFNγ and TNF[37]. Although the exact mechanisms on how these molecules lead to control of bacterial replication are still a matter of debate, it is clear that an effective immune response to fight *M. tuberculosis* involves various cell types, from both the innate and the adaptive immune system. Among these, T lymphocytes, both $CD4^+$ and $CD8^+$ T cells, are crucial for the control of tuberculosis, because of their ability to recognize infected macrophages and directly kill bacteria or by stimulating the macrophage's antimicrobial activity[37].

Although the ability of $CD4^+$ T cells to control mycobacterial infections has been recognized for a long time, the association of AIDS with mycobacterial infections strengthen the clinical importance of these cells[38]. For example, HIV infection has been shown to increase progression from latent to active disease by 5- to 10- fold[38]. Conversely, highly active antiretroviral therapy treatment of HIV patients and the concurrent reconstitution of the $CD4^+$ T cell compartment decrease tuberculosis susceptibility[39]. These human observations are paralleled by studies in the mouse model of tuberculosis, in which mice genetically lacking T and B cells, only T cells, only $CD4^+$ T cells, or wild-type mice depleted of $CD4^+$ T cells are extremely susceptible to infection[40].

Within $CD4^+$ T cells, several distinct subsets have been demonstrated to participate in the control of *M. tuberculosis*. The most well characterized example is that of T_h1 cells, whose development depends on the activation of naïve cells in the presence of IL-12 and on the expression of the signature transcription factor T-bet[41]. T_h1 cells are classically described as producing IFNγ, and accordingly mice unable to

produce IFNγ are extremely susceptible to *M. tuberculosis*[42]. The essential role for IFNγ in the control of mycobacterial infection is also exemplified in people with genetic disorders in the IFNγ pathway, which are extremely susceptible to these infections, in a clinical syndrome described as Mendelian susceptibility to mycobacterial diseases, or MSMD[43]. These two observations, that both IFNγ and $CD4^+$ T cells are essential to the control of *M. tuberculosis*, gave rise to the assumption that IFNγ production by $CD4^+$ T cells is the protective mechanisms underlying *M. tuberculosis* control. However, IFNγ production does not correlate with protection in the context of vaccination[43-45], and in the mouse model, $CD4^+$ T cell mediated protection can be IFNγ independent[46,47]. Also, *M. tuberculosis* modulates MHC-II expression on infected cells, possibly hampering bacterial recognition by $CD4^+$ T cells[48]. These data raise the possibility that IFNγ-independent mechanisms are responsible for the protection mediated by $CD4^+$ T cells, and that other host cells – such as $CD8^+$ T cells, iNKT cells, γδ cells, and other innate cells – might act as the source of IFNγ in vivo.

In addition to T_h1 cells, other $CD4^+$ T cell subsets have been studied during *M. tuberculosis* infection. These include T_h17 cells, T_{regs}, and more recently T_{fh} cells. In this setting, it has been shown that IL-17 production by T_h17 cells improves the recruitment of T_h1 cells to the infected lung[49], and T_{fh} cells are important in granuloma formation[50]. In contrast, the presence of T_{reg} delays the arrival of effector cells to the lung, delaying the establishment of protective immune responses[51]. Although these subsets have been implicated in the response to *M. tuberculosis*, additional studies are necessary to clarify their role in protective immunity and immunopathology, both in animal models and in people.

Like $CD4^+$ T cells, $CD8^+$ T cells have been shown to participate and contribute to immunity against *M. tuberculosis*, in both people and experimental animal models[52] (*$CD8^+$ T cell responses during M. tuberculosis infection are the focus of several studies presented in this thesis, and their contribution to tuberculosis immunity is debated in more extensive detail in Part II of this thesis*). Briefly, mice lacking $CD8^+$ T cells display decreased survival than intact mice[40], and IFNγ production by $CD8^+$ T cells has been shown to promote survival in the absence of $CD4^+$ T cells[53]. In addition to their ability to produce cytokines, $CD8^+$ T cells have been shown to by cytotoxic in both humans and mice[54-57]. However, like for $CD4^+$ T cells, the mechanisms responsible for $CD8^+$ T cell mediated protection in vivo and

their suitability as effective vaccine targets still requires clarification and needs further investigation.

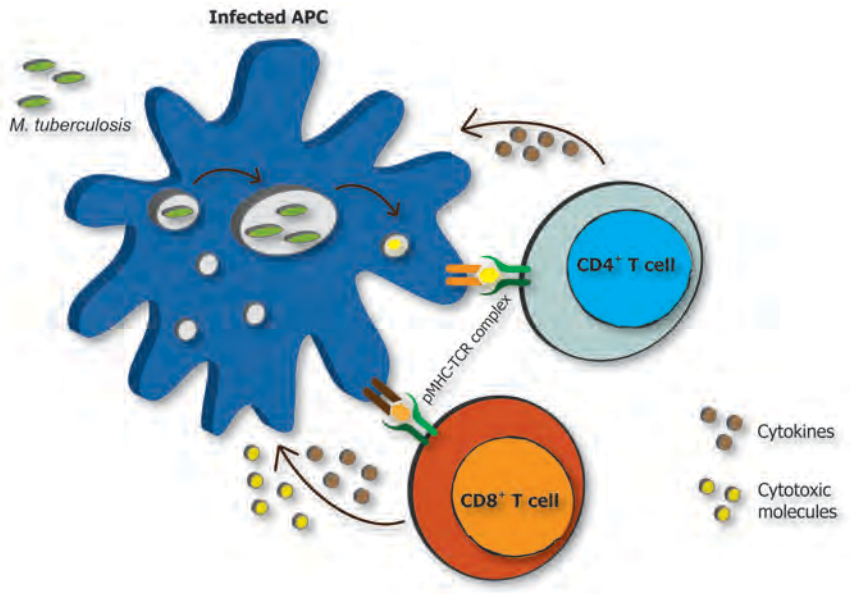

Figure 3. Immune responses to *M. tuberculosis*. Immune responses to tuberculosis involve, among others, macrophages and T cells. Macrophages are essential for bacterial uptake, and present mycobacterial antigens to both $CD4^+$ and $CD8^+$ T cells. Activated T cells produce cytokines, such as IFNγ and TNF, which stimulate the anti-bactericidal mechanisms of macrophages. $CD8^+$ T cells can also be cytotoxic, directly killing infected cells.

Overall, significant improvement has been made in painting the picture of how the immune system deals with *M. tuberculosis* infection, both in terms of the players involved in this fight and the strategies employed by the bug to evade host immunity. However, significant details still need to be elucidated, the most important probably being what correlates with protection against *M. tuberculosis*, which mechanisms do T cells employ to restrict bacterial growth and whether these will be able to promote bacterial clearance. The lack of such knowledge hinders a rationale approach to vaccine development and is a major hurdle in the global fight against tuberculosis.

Scope of the thesis

Although T cells have long been recognized as an indispensable component of the immune response to mycobacteria, there are several questions that remain unaddressed. These include what is the role for $CD8^+$ T cells during *M. tuberculosis* infection, whether they can be protective following infection, and what is their mechanism of protection. Also, despite the key role of the thymus in the generation of T cells, and the observation that mycobacteria disseminate to this organ, it is unclear how the immune system responds to thymic infection and what are the consequences of thymic infection for ongoing immune responses. These are the two areas of investigation discussed in this thesis, and we aim to:

In part I - the problem of thymic infection:

- clarify how the immune system controls thymic infection, and what cells are responsible for bacterial control;
- investigate the mechanisms by which immune cells are recruited to the infected thymus.

In part II - $CD8^+$ T cell responses to tuberculosis:

- generate a new mouse model expressing $CD8^+$ T cells specific for TB10, an immunodominant *M. tuberculosis* antigen;
- investigate the protective capacity of $CD8^+$ T cells during *M. tuberculosis* infection;
- dissect the mechanism(s) responsible for $CD8^+$ T cell mediated protection.

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The first section of this thesis compiles studies on thymic infection by mycobacteria. It starts with a review on the problem of thymic infection, currently submitted for publication. This is followed by a manuscripts published in *The Journal of Immunology* entitled "*T cells home to the thymus and control infection*", where we show how the thymus is able to control *M. avium* and *M. tuberculosis* infection. Together, these studies help shape the notion of the thymus as a target of infection, and that thymic infection as important consequences on ongoing immunity

Part I

The problem of thymic infection

how the immune system controls thymic infection, and the consequences of thymic infection on T cell differentiation

For a long time, the thymus was considered an immune privileged site, protected from any changes occurring in other organs of the body, such as during infectious episodes. However, recent data has shown that infection impacts thymic structure and function. These modifications occur either through pro-inflammatory mediators that accompany systemic infection, or by direct infection of the thymus by a variety of pathogens, including bacteria, viruses, fungi and parasites. In this chapter, we review how infection affects thymic structure and function and discuss the consequences of alterations in thymic activity on ongoing immunity.

Chapter 2

Tolerance has its limits: how the thymus copes with infection

A review on the consequences of infection on thymic function

(This manuscript is currently under revision)

Tolerance has its limits: how the thymus copes with infection

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Abstract

The thymus is required for T cell differentiation, a process that depends on which antigens are encountered by thymocytes, the environment surrounding the differentiating cells, and the thymic architecture. These features are altered by local infection of the thymus and by the inflammatory mediators that accompany systemic infection. Although once believed to be an immune privileged site, recent studies show that anti-microbial responses are recruited to the thymus. Resolving infection in the thymus is important because chronic persistence of microbes impairs the differentiation of pathogen-specific T cells and diminishes resistance to infection. Understanding how these mechanisms contributes to disease susceptibility, particularly in infants with developing T cell repertoires, requires further investigation.

Introduction

The appearance of adaptive immunity in jawed vertebrates is considered a major evolutionary step, as B and T cells enable the immune system to generate and recall pathogen-specific immune responses[1]. Among lymphocytes, T cells are unique in their expression of the T cell receptor (TCR). TCR-mediated recognition of microbial peptides bound to major histocompatibility complex (MHC) is the principle way that the immune system identifies infected cells. T cell precursors are generated in the bone marrow and become functional after differentiation within the thymus[1]. The deceptively simple anatomical structure of the thymus belies its sophisticated ability to generate T cells expressing a broad TCR repertoire capable of recognizing virtually any foreign antigen. Importantly, thymic selection eliminates most potentially harmful self-reactive T cells. After selection, naïve self-restricted T cells exit the thymus and traffic to secondary lymphoid organs.

T cell differentiation depends on the thymic microenvironment and the cytokine milieu surrounding the differentiating cells[1]. This raises the possibility that during infection, changes in soluble factors or antigens present within the thymus alters T cell differentiation. Indeed, recent reports show that systemic infection has detrimental effects on thymus structure and function. Furthermore, certain bacteria, virus, fungi and parasites can directly invade the thymus (Table 1). These observations suggest that some pathogens, particularly the ones that cause chronic infections, interfere with the generation of immune responses designed to fight them by disrupting T cell development and possibly altering central tolerance.

Thymus: myth and reality

Altered thymic function is reported during infection in animal models. However, with the exception of studies on human immunodeficiency virus (HIV) infection[2], few studies have addressed whether infection affects thymic function in people. In part, this is because of limited availability of human samples, since the thymus is difficult to biopsy. As important are misconceptions that have affected how scientists and physicians look at thymic function. Although now largely refuted, these include: (1) the thymus is an immune-privileged site protected from infection and immune responses; and (2) thymic function is only important during early life and dispensable after puberty.

The notion that the thymus is immune privileged is inseparable from the concept of the blood-thymus barrier, considered for several years responsible for an antigen-free thymic microenvironment and no ongoing immune responses[3]. However, it is now clear that the

thymus is both a target of infection and a site to where immune responses are recruited (Table 1)[4-7].

While the thymus is essential for the establishment of a diverse T cell repertoire early in life, it is widely believed to be unnecessary after puberty. This idea is supported by finding that the peripheral T cell pool can be maintained by thymic-independent mechanisms[8]. Even if this were true, it is incredibly shortsighted to ignore the effect of infection on the thymus, as children and young adults under the age of 24 make up 44% of the world's population and are particularly vulnerable to infection. In 2011, nearly 6.9 million children under the age of five died, with infection causing more than half of these deaths[9]. Although most of these deaths were due to acute infection (pneumonia, 14%; diarrhea, 10%; measles, 1%), a significant number were due to chronic infection (malaria, 7%; HIV/AIDS, 2%; tuberculosis, 1%)[9]. These numbers increase with age and HIV/AIDS and tuberculosis account for 11% of deaths among young adults (10-24 years) worldwide[9]. Interestingly, both HIV and *Mycobacterium tuberculosis* infect the thymus and cause alterations in T cell output, which could be relevant both in settings of vaccination and during natural immunity to these pathogens[2,10,11].

Although childhood infections are arguably a greater cause of morbidity and mortality than adult infection, particularly in the developing world, recent studies reinforce the idea that the thymus affects resistance to infection during adulthood. Reduced thymic output of T cells is associated with HIV progression to AIDS and the thymus has been implicated in the successful immune reconstitution of AIDS patients in response to antiretroviral therapy[2]. Moreover, thymectomy during early childhood has been linked to accelerated decline in immunologic function, particularly following cytomegalovirus (CMV) infection[12], and work on experimental viral infection models finds that continuous recruitment of naïve T cells from the thymus has a beneficial role in the control of persistent infections[13-15]. The integrity of the adult thymus is also required for other aspects of ongoing immunity including antibody generation[16] and oral tolerance[17]. These observations indicate that an intact and functional thymus is required for optimal immunity to infection throughout life.

How does infection alter thymic function?

There are two ways in which infection can affect the thymus: ***local*** versus ***systemic***. ***Local*** refers to effects of direct infection of the thymus by microbes. ***Systemic*** refers to the consequences of infection somewhere else on the thymus. Systemic effects occur when soluble factors, such as glucocorticoids (GC) and other pro-inflammatory mediators, are released into the blood stream.

Infection-induced thymic atrophy

Premature thymic atrophy is a common consequence of infection by viruses, bacteria, parasites and fungi (Box 1)[18] and can be a ***local*** and/or ***systemic*** effect. For example, GC levels rise during acute infection, and can induce thymocyte apoptosis, especially among double-positive (DP) thymocytes[19]. Adrenalectomy prior to infection prevents thymocyte depletion in rabies virus infected mice, which confirms a role for GCs in infection-induced thymic atrophy[20]. Infection-induced premature thymic atrophy also occurs independently of increased systemic GC levels. For example, adrenalectomy prior to *Toxoplasma gondii* infection abolishes peripheral lymphopenia but does not prevent thymocyte loss[21]. In other infections, GC synergize with other mediators to induce thymic atrophy. These include TNF during *Francisella tularensis*[22] and *Trypanosoma cruzi* infection[23], IFNγ during *Salmonella enterica* infection[24], and IFNγ and nitric oxide during *Mycobacterium avium* infection[25].

Interestingly, infection-induced thymic atrophy often correlates with strain virulence, as observed for *T. cruzi*[18], *F. tularensis*[22], *M. avium*[25], measles virus[26], highly pathogenic avian influenza viruses (HPAIV)[7] and simian immunodeficiency virus (SIV)[27]. These data suggest that specific microbial factors directly promote thymocyte death. This is true for bacterial factors such as LPS[28], *Escherichia coli* enterotoxin[29] and mycobacterial cord factor[30] and has been confirmed with the fungal virulence factors gliotoxin[31] and toxin T-2[32], all of which directly induce thymocyte apoptosis when administered to mice. Local thymic effects are also observed after HIV infection[2]. Interestingly, HIV thymotropic viral variants are detected *in vivo*[33], raising the possibility that these strains would be more likely to affect thymic function. The infected cell type depends on viral tropism for CXCR4 and CCR5, but most thymocyte subsets can become infected[2]. HIV also infects different thymic stromal cells, including macrophages, both conventional (cDC) and plasmacytoid DC (pDC) – and thymic epithelial cells (TEC)[34-36]. HIV affects the fate of infected cell types differently. For example, CD4SP thymocyte depletion results from direct infection, killing of progenitor cells, and apoptosis induction of uninfected cells by viral products[37]. HIV infection also induces DC and TEC death[34,36]. In the case of DC, cell death is associated with IFNα production by pDC but not cDC[34]. Thus, in addition to inducing thymocyte death, HIV disrupts thymic function by altering the local microenvironment. Collectively, these data show that HIV infection induces thymic atrophy and impacts thymic function, leading to a decline in the export of newly differentiated T cells[2,38].

Similar to HIV, murine leukemia virus (MLV) has specific LTR region sequences that affect viral infection and replication in DP and DN thymocytes and in other thymic populations[39]. MLV induces apoptosis of infected cells within the thymus even before the

leukemic period. Apoptosis is induced by the accumulation of Env protein precursors, triggering endoplasmic reticulum stress[40]. Thus, apoptosis is a consequence of local infection, and infected DP cells die more than uninfected cells within the thymus.

Thymic atrophy is also detected during infection with HPAIV[7]. These viruses cause severe human disease accompanied by profound lymphopenia. After intranasal challenge of mice, influenza-infected DC are present in the cortico-medullary region and medulla of the thymus. Thymic atrophy is strain dependent and occurs only after infection with highly pathogenic virus. HPAIV interferes with thymic function, inducing loss of DP thymocytes and diminished export of naïve T cells to the periphery, leading to severe lymphopenia. It is unknown what role GCs play during this acute infection and the relative contribution of local versus systemic factors to thymic atrophy cannot be ascertained.

These studies demonstrate that local and systemic effects can induce thymic atrophy during infection, and are not mutually exclusive.

Thymic structure is altered by infection

Infection induces thymic structural alterations other than atrophy. For example, both *T. cruzi* and *Plasmodium berghei* directly infect the thymus and induce significant changes in the extracellular matrix[18,41-43]. *T. cruzi* increases fibronectin and laminin deposition and CXCL12 and CCL4 production within the thymus[43,44]. During *T. cruzi* infection, expression of the fibronectin and laminin receptors (VLA-4, VLA-5 and VLA-6) and CXCR4 and CCR5 is augmented on thymocytes and intrathymic thymocyte migration of DP cells is enhanced[43,44]. *P. berghei* affects thymocyte migration by inducing CXCL12 and CXCR4 and reducing CCL25 and CCR9 production within the thymus[42]. When analyzed *ex vivo*, both DN and SP cells from infected thymi migrate faster than control populations towards extracellular matrix components[42]. In both cases, these changes affect peripheral T cell subsets. At the peak of *T. cruzi* infection, a higher frequency of immature and VLA^{hi} DP T cells are found in the periphery[43]. Similarly, increased numbers of DN and DP T cells are found in the periphery of *P. berghei* infected mice[41].

Viral infections also induce significant changes in thymic structure by infecting cells in stromal cells[45]. HIV can infect TEC and lead to degeneration of these cells[36]. Hepatitis virus[46], MLV[47], measles virus[48], CMV[49] and type-B Coxsackievirus (CV-B)[50] also infect TEC. Interestingly, *in vitro* infection of human TEC with measles virus results in terminal differentiation and apoptosis of these cells[48]. In contrast, *in vitro* CV-B infection of human TEC does not cause damage but modulates cell function, leading to increased production of IL-6, GM-CSF and leukocyte migration inhibition factor (LIF)[50]. The observation that viruses infect TEC, alter their function, and in some cases induce cell death, is especially important

given the crucial role these cells play in T cell development[51]. Finally, the changes in thymic cellularity observed during viral infection may be caused by depletion of TEC and secondary decline in thymocyte number.

These data show that structural alterations of the thymus caused by infection modify the characteristics of differentiating T cells and affect T cell export.

Alterations in thymic export

The appearance of DP thymocytes in the periphery, as described following *T. cruzi* and *P. berghei* infection, is also a hallmark of infections caused by HIV, hepatitis B (HBV) and hepatitis C (HCV) virus[52]. These viruses can be detected within the thymus (Table 1), and although the origin of peripheral DP cells is unknown, one possibility is that they are released from the thymus after local infection. Alternately, activated T cells may acquire a DP phenotype as observed during HIV and other infections[53]. Whether peripheral DP cells are an indication of altered thymic function or whether they represent activated T cells likely varies according to the infecting agent and requires experimental determination.

Alterations in thymic structure due to infection can alter T cell export in other ways. A major consequence of HIV infection is reduced export of recent thymic emigrants, an effect confirmed by T cell rearrangement excision circles (TREC) analysis[2,38]. Since thymic activity is essential to maintain or reconstitute a functional peripheral T cell pool, interventions that enhance this process may potentiate the beneficial effects of anti-retroviral therapy on immunity[54].

Although it's intuitive that reduced thymic T cell export is a consequence of infection-induced thymic atrophy, this is not always the case. During *Salmonella*-induced thymic atrophy, T cell export is maintained[55], suggesting that atrophy and a decline in thymic function might be independent. Nevertheless, maintaining thymic export of recent thymic emigrants during infectious episodes is important, as it positively impacts ongoing immunity[13-17].

Generation of T cells tolerant to pathogens

We've shown how infection alters thymic structure and affects export of immature and naïve T cells. Another important question is whether infection of the thymus and the presence of microbial antigens in the thymus, particularly during persistent infection, leads to pathogen-specific tolerance. This question was investigated using a model of mycobacterial infection. Infection of mice with *M. avium* of intermediate virulence leads to chronic persistent infection. During this infection, *M. avium* disseminates to, and persists in, the

thymus[25,56]. No thymic atrophy occurs and the chronically infected thymus continues to support T cell differentiation. However, T cells that mature in infected thymi are abnormal as they suboptimally respond to *M. avium* antigens compared to T cells that differentiate in uninfected thymi. Importantly, T cells that differentiate in *M. avium* infected thymi respond normally to unrelated antigens, indicating that the defect is specific for the invading pathogen[11]. Although the precise mechanism(s) responsible for this difference is still being elucidated, these data demonstrate that thymic infection can induce pathogen-specific T cell tolerance.

Tolerance to invading pathogens is also observed during viral infection. Neonatal MLV infection leads to infection of thymocytes as well as thymic stromal cells and renders T cells tolerant to virus antigens[47]. Similarly, congenitally acquired LCMV infection is a model of immune tolerance: mice infected *in utero* or at birth show high viral titers in most organs, including the thymus, and have a selective defect in LCMV-specific T-cell immunity[57]. LCMV infection of the thymus starts at the fetal stage in DN cells, and transitions to $CD4^+$ T cells in the adult thymus. In contrast, $CD8^+$ T cell infection is minimal[58], and transferred virus-specific cytotoxic T lymphocytes (CTL), from immunized mice, infiltrate the thymus and eliminate the infection[6,57]. In this model, viral clearance is associated with reacquisition of LCMV-specific CTL responses, suggesting that continuous presence of the antigen in the thymus is required to maintain tolerance, not only during fetal development but in adult animals as well.

Viral hepatitis also induces central tolerance to HBV. While acute infection in adults is readily resolved, HBV infection *in utero* induces tolerance to viral proteins, and infants born to HBV-infected mothers are more likely to become chronic carriers of HBV[59,60]. One explanation is that viral proteins in the neonatal thymus induce HBV-specific T cell tolerance. Significantly, both MLV and HBV infect TEC[46,47], which could explain why these infections induce T cell tolerance while others do not. Since TEC turnover is rapid[61] this hypothesis implies that these viruses can continually infect new TEC or reside in thymic epithelial stem cells.

These data suggest that direct infection of the thymus by virus and bacteria alter T cell selection and induce tolerance against the invading pathogen, with the potential to impair ongoing immunity. An interesting question is whether the mechanism of tolerance induced by thymic infection involves the generation of pathogen-specific regulatory T cells, T cell anergy or negative selection of differentiating pathogen-reactive T cells.

Autoimmunity induced by thymic infection

Pathogens that disrupt thymic function may also disrupt the development of central tolerance. Autoreactive T cells that are normally negatively selected in the thymus might

escape death because of thymic dysfunction, emigrate to the periphery, and trigger autoimmunity. While this possibility remains theoretical, some data supports it. During experimental *T. cruzi* infection, T cells expressing ‘‘forbidden’’ TCRs that are normally deleted in the thymus, particularly those belonging to the Vβ5 and Vβ12 families, survive and can be detected in peripheral LNs. Importantly, thymic SP cells from infected mice are not enriched in those TCRs. These data indicate that the appearance of these “prohibited” TCRs in the periphery is not due to defective negative selection but a consequence of abnormal migration of immature cells[62]. In addition, anti-thymus antibodies and myocardium-specific autoreactive T cells are detected following *T. cruzi* infection[18], raising the possibility that infection-induced thymic alterations potentiate autoimmunity.

Thymic infection: the beginning and the end

We next turn our attention to how these various microbes are able to reach the thymus and establish infection. Furthermore, if dissemination to the thymus occurs commonly during infection, has the immune system evolved mechanisms to respond to pathogens invading the thymus?

How do microorganisms reach the thymus?

Two scenarios are possible when considering the origin of thymic infection during hematogenous spread of infection. First, circulating pathogens can enter the thymus and infect cells in a targeted manner, as represented by thymotropic variants of HIV[33] and MLV[39]. Alternately, there is the “Trojan Horse” model. The trafficking of several cell types between the periphery and the thymus make this possible. T cells re-circulate from the periphery to the thymus[63], and if infected, could seed the thymus with pathogens that target T cells (e.g., HIV). Similarly, certain DC subsets (e.g., Sirpα$^+$ cDCs) migrate from the periphery to the thymus and modulate T cell tolerance[64]. Interestingly, DC infected with influenza virus or *M. avium* can be detected within thymus, raising the possibility that infected DC spread the infection[7,11]. DC are responsible for disseminating *M. tuberculosis* from the lung to the draining lymph node[65], and it is possible that dissemination to the thymus occurs by a similar mechanism. Together, these data support the idea that cells circulating to the thymus can carry infectious agents and seed thymic infection. An important corollary is that the route and duration of infection may modify the risk that the thymus becomes infected by different pathogens.

Immunity within the thymus

If the thymus is a site of infection, it may recruit an immune response against invading pathogens. As discussed, during systemic LCMV infection, LCMV-specific CTL traffic to the thymus, establish an immune response, and eliminate LCMV from the thymus[6,57]. Similarly, during influenza infection, functional influenza-specific CTLs are detected within the thymus[7]. Finally, antigen-specific $CD4^+$ and $CD8^+$ T cells are detected in the thymus following *M. avium* and *M. tuberculosis* infection, as part of the immune response against persistent bacteria[4,5]. Importantly, the responding T cells in the thymus are not newly differentiated mature thymocytes but instead are activated T cells that recirculate from peripheral organs to the thymus to fight infection[4]. Under these conditions, the recruitment of activated T cells is associated with increased expression of T helper 1 chemokines and an enrichment of $CXCR3^+$ mycobacteria-specific T cells within the thymus[4]. These results confirm that the thymus is not only a site of infection, but suggest that it is actively surveyed by the immune system.

Final remarks

The recent studies reviewed here show that the thymus is a site of infection that has important immunological consequences. Pathogens disrupt thymic structure and function, and alter T cell selection and export. These changes affect the peripheral T cell pool and affect ongoing and future immune responses.

These data suggest a model in which the effect of infection on the thymus depends on the type of microbe, the severity of infection and the ability of the pathogen to infect and persist within the infected thymus (Figure 1). In this scheme, acute infection is characterized by increased GC and pro-inflammatory mediator levels, which can lead to thymic atrophy. This effect is more pronounced on DP cells and can occur even in the absence of the pathogen in the thymus. Local thymic infection can exacerbate atrophy, through remodeling of extracellular matrix, production of virulence factors or direct infection of thymic cells. These structural changes affect thymic function; particularly T cell export, leading to the release of immature (DP/DN) or autoreactive T cells into the periphery. Despite the profound effects of acute infection on the thymus, the impact of thymic dysfunction on immunity is predicted to be limited and transient as the peripheral T cell pool should include pre-existing pathogen-specific T cells. In contrast, local thymic infection may have severe repercussions, particularly for: 1) infections acquired during childhood – when the T cell repertoire is still developing; 2) in the setting of persistent infection (e.g., tuberculosis), which may induce to T

cell tolerance; 3) infections associated with severe lymphopenia (e.g., HIV), when lymphoid reconstitution is required. In these cases, emergence of central tolerance to the infectious agent may impair deployment of pathogen-reactive T cells in the naïve repertoire. Such a scenario could favor the microbe, since impairment of T cell immunity would contribute to pathogen persistence. In order to minimize such consequences, mechanisms exist to respond to direct infection of the thymus (Figure 2). Just as in other tissues, these rely on the trafficking of peripheral T cells from secondary lymphoid tissue back to the thymus. While required for protection, circulation of cells back to the thymus could allow some pathogens access to the thymus.

Altogether, preserving a sterile thymic environment is essential to maintain thymic integrity, both structurally and functionally, sustain optimal T cell differentiation and export, and prevent the emergence of tolerance to invading pathogens. Therefore, the thymus should be regarded as an active player during infectious episodes and the contribution of this organ for ongoing immunity should be addressed in future studies (Box 2).

Tolerance has its limits

Disclosures

The authors have declared that no conflict of interest exists.

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Boxes

Box 1. Mechanisms of thymic atrophy.

Thymic atrophy, or involution, refers to the decrease in size of the thymus, and usually correlates with a decline in thymic function, resulting in a reduced export of naïve T cells to the periphery. Although involution is a physiological process, and the thymus normally atrophies with aging and pregnancy, several non-physiological stimuli can induce thymic atrophy, such as stress and infection. Infection-induced atrophy occurs by at least two different mechanisms: alterations in TEC or induction of apoptosis in thymocytes, particularly DP cells. Why thymic atrophy should accompany infection is debated. The different hypotheses include: (1) thymic atrophy is a by-product of infection, with no specific advantage for the pathogen or the host; (2) thymic atrophy is a virulence strategy employed by pathogens to subvert antimicrobial immunity; and (3) thymic atrophy is a host strategy that reduces thymic activity during infection to prevent disruption of T cell selection and prevent the emergence of central tolerance to the invading organism. In any case, thymic atrophy can impair thymic function and has implications for ongoing immunity.

Box 2. Outstanding questions.

How universal is thymic infection? Do other pathogens infect the thymus?

How does the thymus get infected? Do pathogens directly target the thymus, or do they disseminate inside recirculating cells?

Why does atrophy accompany thymic infection so frequently? Is atrophy beneficial for the host or the bug?

What are the mechanisms responsible for T cell tolerance? Does the presence of microbial antigens lead to negative selection of developing T cells? Do microbe-specific Tregs emerge following thymic infection? Are developing T cells anergic to the infectious agent?

How does infection of the thymus impact ongoing immunity? Does thymic infection during childhood impact immunity later in life? Is thymic infection relevant during vaccination?

How important are newly generated T cells during chronic infections and during immune reconstitution?

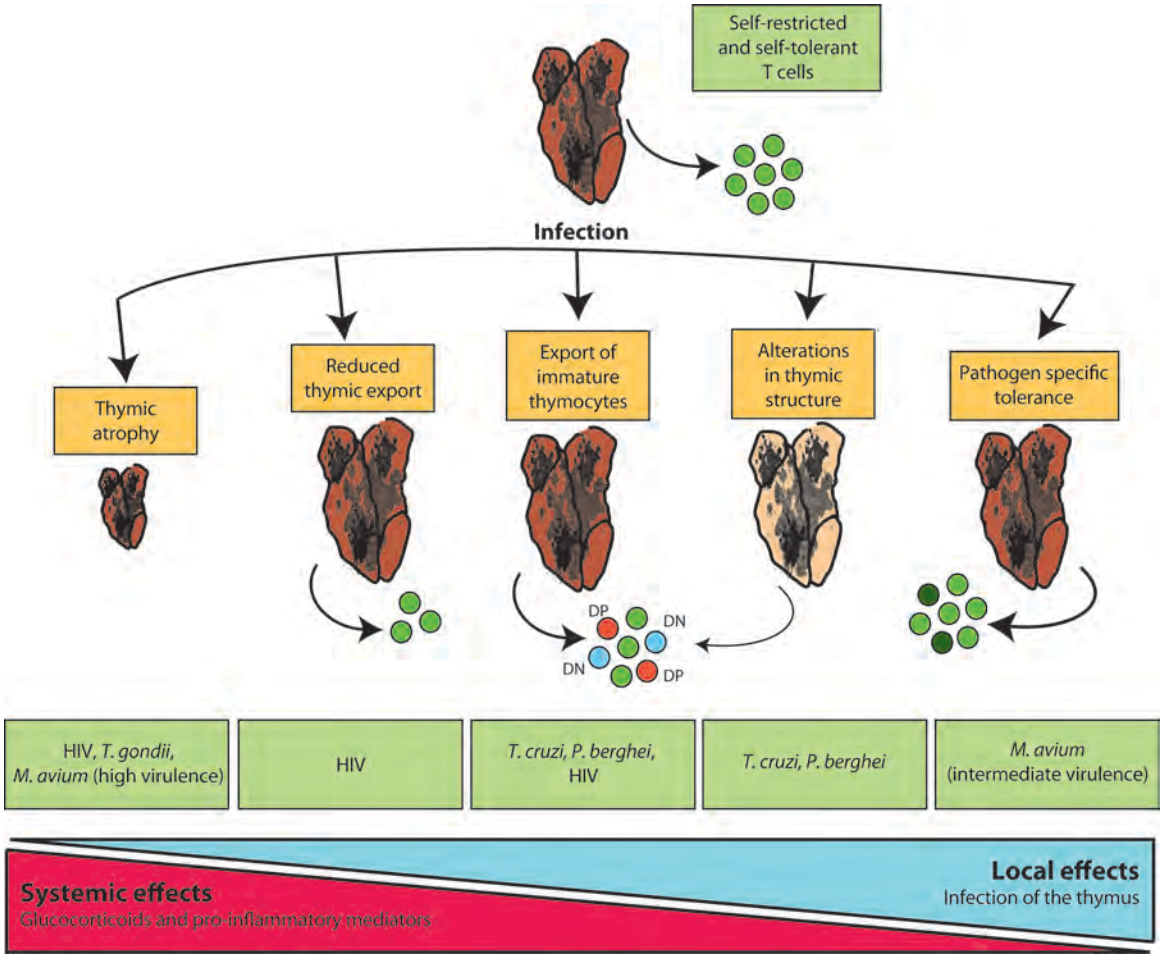
Figures

Figure 1. The effects of infection on the thymus. Schematic representation of how infection can affect the thymus through systemic and/or local effects. Glucocorticoids and/or pro-inflammatory mediators mediate systemic effects, while local effects require the presence of a pathogen within the thymus. Infection-induced alterations include thymic atrophy, modifications in the thymic structure and alterations in the T cells exported to the periphery. Representative pathogens capable of inducing the different alterations in thymic structure and/or function are indicated. DN – double negative. DP – double positive.

Figure 2. Immune response in the thymus. Schematic representation of microbial dissemination and recruitment of an immune response to the thymus. Under normal conditions, mature T cells and DC re-circulate from peripheral lymphoid organs to the thymus (*left panel*). Following infection, pathogens disseminate from the periphery to the thymus, either extracellularly or within re-circulating cells. The infected thymus produces chemokines, such as CXCL9 and CXCL10, which recruit CXCR3-expressing antigen-specific T cells from the peripheral tissues back to the thymus to fight infection (*right panel*). LN – lymph node.

Table 1. Pathogens that infect the thymus. Virus, bacteria, fungi and parasites have been documented to infect the thymus, either in humans or experimental animal models. The major consequences of each infection on thymic structure or function are listed, when available.

Figures, tables and legends



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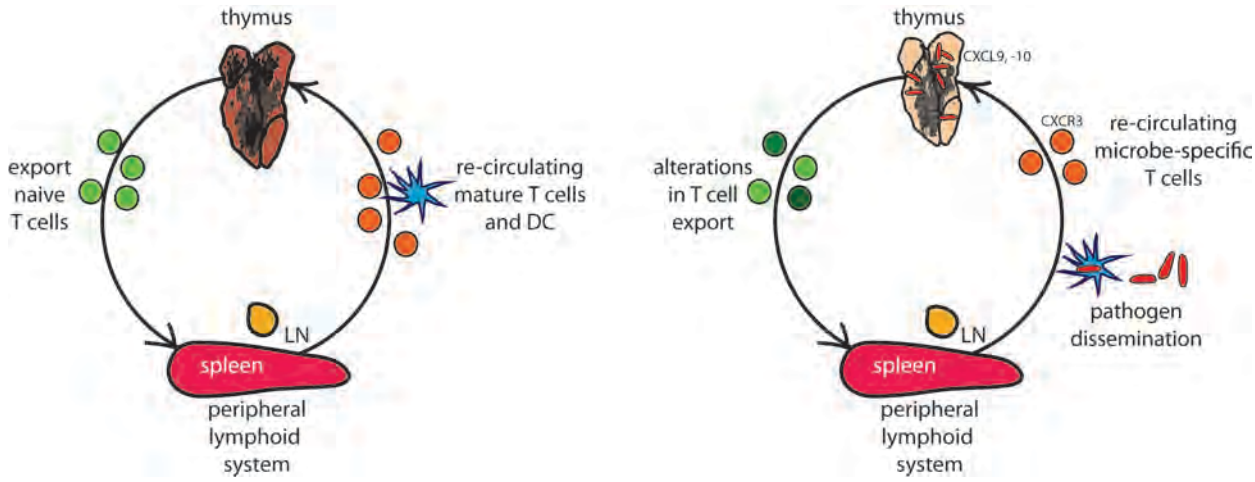

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Table 1: Pathogens that infect the thymus

	Pathogen	Consequences of infection	References
Virus	Human Immunodeficiency virus (HIV)	Atrophy; DP cells in the periphery	5, 6, 22, 62-64, 67
	Simian Immunodeficiency virus (SIV)	Atrophy (*strain dependent*)	37
	Influenza virus	Atrophy (*strain dependent*); immune response in the thymus	17
	Lymphocytic Choriomeningitis virus (LCMV)	Atrophy; immune response in the thymus; immune tolerance	16, 80, 81, 82, 83, 85
	Murine Leukemia virus (MLV)	Atrophy; immune tolerance	69, 70, 79
	Hepatitis virus	Atrophy; immune tolerance; DP cells in the periphery	73, 74, 86, 100
	Murine Cytomegalovirus (MCMV)	Atrophy	36
	Measles virus	Atrophy (*strain dependent*)	55, 102
	Epstein-Barr virus (EBV)	N/A	99, 105, 106
	Junin virus	N/A	97
	Poliovirus	N/A	98
	Pycornavirus	N/A	93, 94, 101
Bacteria	*Mycobacterium avium*	Atrophy (*strain dependent*); immune response in the thymus; immune tolerance	14, 23, 51, 54, 78
	Mycobacterium tuberculosis	Immune response in the thymus	14, 15, 21, 78
	Francisella tularensis	Atrophy (*strain dependent*)	47, 48
	Salmonella enterica	Atrophy	50, 77
Fungi	*Paracoccidioides brasiliensis*	Atrophy	95, 104
	Cryptococcus neoformans	Alterations in thymic architecture	103
Parasites	*Trypanosoma cruzi*	Atrophy (*strain dependent*); release of DP/DN/autoreactive T cells; alterations in extracellular matrix	32, 35, 39, 40, 49, 53, 72, 88, 91
	Plasmodium berghei	Atrophy (*strain dependent*); release of DP/DN cells; alterations in extracellular matrix	33, 34
	Toxoplasma gondii	Atrophy	52, 96

Table 1. Pathogens that infect the thymus. Virus, bacteria, fungi and parasites have been documented to infect the thymus, either in humans or experimental animal models. The major consequences of each infection on thymic structure or function are listed, when available. DN – double negative. DP – double positive.

In chapter 2, we review data on multiple microbes that infect the thymus and alter thymic structure and function. However, if dissemination to the thymus is a common consequence of infection, with potentially severe implications for ongoing immunity, the immune system must have evolved mechanisms to control or contain the invading pathogens. That is the subject of this chapter, in which we report how the immune system recruits peripheral T cells back to the thymus in order to mount immune responses that control mycobacterial infection in this organ.

Chapter 3

T cells home to the thymus and control infection

How the thymus controls mycobacterial infection

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T cells home to the thymus and control infection

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Abstract

The thymus is a target of multiple pathogens. How the immune system responds to thymic infection is largely unknown. Despite being considered an immune privileged organ, we detect a mycobacteria-specific T cell response in the thymus following dissemination of *Mycobacterium avium* or *Mycobacterium tuberculosis*. This response includes pro-inflammatory cytokine production by mycobacteria-specific $CD4^+$ and $CD8^+$ T cells, which stimulates infected cells and controls bacterial growth in the thymus. Importantly, the responding T cells are mature peripheral T cells that recirculate back to the thymus. The recruitment of these cells is associated with an increased expression of T_{h1} chemokines and an enrichment of $CXCR3^+$ mycobacteria-specific T cells in the thymus. Finally, we demonstrate it is the mature T cells that home to the thymus that most efficiently control mycobacterial infection. Although the presence of mature T cells in the thymus has been recognized for some time, these data are the first to show that T cell recirculation from the periphery to the thymus is a mechanism that allows the immune system to respond to thymic infection. Maintaining a functional thymic environment is essential to maintain T cell differentiation and prevent the emergence of central tolerance to the invading pathogens.

Introduction

The appearance of adaptive immunity in jawed vertebrates is considered a major step in the evolution of the immune system, as it provides the host with a crucial tool to combat microbial pathogens: namely B and T lymphocytes [reviewed in (1, 2)]. B and T lymphocytes express antigen receptors that result from the recombination of germline encoded gene segments, which establishes a diverse repertoire of antigen receptors. This allows B and T cell antigen receptors to recognize antigens the host has not previously encountered. The TCRs, which recognize short linear peptide fragments in the context of antigen presenting molecules (class I or II MHC), allow the host to survey both the intra- and extracellular environment.

The diverse repertoire of TCRs, which are generated by genetic recombination, creates specific challenges for the immune system. First, not all TCRs generated will recognize self-MHC/peptide complexes. Second, some TCRs will recognize self-MHC/peptide complexes with such a high affinity/avidity that will render them potentially autoreactive. To avoid full differentiation of T cells with these unwanted characteristics, thymocytes go through a complex process of selection during their differentiation in the thymus. T cell precursors emerge from the bone marrow and home to the thymus, a primary lymphoid organ that exists for the purpose of supporting T cell differentiation. Two fundamental selection processes occur during T cell differentiation in the thymus – positive and negative selection [reviewed in (3)]. The outcome of thymic selection depends on the organ microenvironment including which antigens are present within the thymus and the cytokine milieu surrounding the differentiating cells. Positive selection creates a pool of thymocytes that bind self-MHC/peptide complexes while T cells expressing TCRs that fail to bind these complexes undergo cell death by apoptosis. T cells expressing TCRs that bind self-MHC/peptide complexes with high affinity/avidity are also eliminated through negative selection. This process of central tolerance is essential to generate a repertoire of T cells that is self-restricted and self-tolerant, and which limits the development of potentially autoreactive T cells. Thus, proper thymic activity is crucial for the development and maintenance of a repertoire of functional T cells. Infants born with genetic mutations that abrogate T cell differentiation are profoundly susceptible to infection, which confirms the essentiality of T cells for cell-mediated immunity (4).

Thymic activity decreases naturally with age, although the adult thymus still supports T cell differentiation and T cells continue to be exported into the peripheral T cell pool (5, 6). Coincident with T cell differentiation and thymic activity, the developing immune system is assaulted by various microbial pathogens. In fact, thymic function is affected during infection by several pathogens, including species of bacteria, virus, fungi and parasites (7, 8). Both in

humans and animal models the thymus is itself a target of infection (7-19). Infection of the thymus by mycobacterial species has been reported both in humans (16) and in animal models (17-19). The incidence of thymic infection during active tuberculosis is unknown although there are several case reports in the clinical literature (16). However, it is relevant that vertical transmission of tuberculosis (mother to child) frequently occurs during early childhood, at the height of thymic activity (20).

Although the thymus has been formerly considered an immune privileged site (21), this idea is being reconsidered. In fact, it is clear that protection of the thymus from infection, particularly during childhood, a time of diverse and recurrent infection, should be a function of the immune system. Since negative selection of T cells depends on the antigens encountered in the thymus during differentiation, infection of this organ could theoretically lead to the development of immune tolerance to pathogens. How the immune system prevents, or combats thymic infection, to maintain the organ's integrity and function, and keep it free from microbial antigens is still unclear. Interestingly, the thymus does contain several populations of mature T cells. These include innate lymphocytes such as iNKT cells (22), γδ T cells (23), and MR1-restricted MAIT cells (24), all of which have been implicated in host defense against infection. In addition, there are recirculating conventional $CD4^+$ and $CD8^+$ T cells (25). Why mature T cells should recirculate back to the thymus is unknown, although others have hypothesized that these T cells play a role in surveying the thymus for infection [reviewed in (25-27)].

Despite the introduction of BCG vaccination a century ago and the development of multiple pharmacological drugs that are active against mycobacteria, these bacteria are still one of the most prevalent infectious agents worldwide (28). Among these, *Mycobacterium tuberculosis* alone is estimated to cause approximately 8.8 million new infections and 1.4 million deaths per year (28). In addition to *M. tuberculosis*, other members of the *Mycobacterium* genus including *Mycobacterium marinum*, *Mycobacterium ulcerans, Mycobacterium leprae* and *Mycobacterium avium* cause disease in humans (29). Some of these are opportunistic infections and mainly affect immunocompromised individuals (30). In fact, the spread of HIV dramatically increased the prevalence of active mycobacterial infection, which is the main cause of death in patients with AIDS (31).

We previously showed that experimental infection with *M. tuberculosis* and *M. avium* leads to the establishment of thymic infection in chronically infected mice (18, 19). Now we report that infection of the thymus is followed by the establishment of protective immunity within the thymus, characterized by the appearance of both $CD4^+$ and $CD8^+$ T cells specific for mycobacterial antigens. These antigen-specific T cells do not originate from the pool of differentiating T cells in the thymus, but are instead T cells that recirculate from peripheral organs back to the infected thymus to control infection. Their recruitment to the thymus

correlates with the expression of the chemokine receptor CXCR3 and the production of CXCL9 and CXCL10 by the infected thymus. These data are the first to show that T cell recirculation to the thymus is a mechanism used by the immune system to survey and protect the thymus from infection and maintain thymic integrity.

Materials and Methods

Mice and infection

C57BL/6 (WT) mice were purchased from Charles River Laboratories (Barcelona, Spain) or from Jackson Laboratories (Bar Harbor, ME) and CD45.1 mice (B6.SJL-Ptprca Pepcb/BoyJ)(32) and TCRα KO (B6.129S2-Tcra^{tm1Mom}/J) from The Jackson Laboratory (Bar Harbor, ME)(33). RAG-GFP mice (34) were kindly provided by Dr. António Bandeira (Pasteur Institute, Paris, France). Both TCRα KO and RAG-GFP mice were bred in our facilities. Mice were 7 to 10 weeks old at the start of the experiments. All animal experiments were performed in accordance with National and European Commission guidelines for the care and handling of laboratory animals and were approved by the National Veterinary Directorate and by the local Animal Ethical Committee or by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines. Mice infected with *M. tuberculosis* were housed in a biosafety level 3 facility under specific pathogen-free conditions at the Animal Biohazard Containment Suite (Dana Farber Cancer Institute, Boston, MA).

Experimental infection

M. avium (strain 2447, provided by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium) infection was performed intravenously through the lateral tail vein delivering 10^6 CFU per mouse. For each *M. tuberculosis* (Erdman strain) infection, a bacterial aliquot was thawed, sonicated twice for 10 s in a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. A 15 ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies) and mice were infected via the aerosol route using a nose-only exposure unit (Intox Products) and received 100–200 CFU/mouse. At different times post-infection, mice were euthanized by carbon dioxide inhalation or by decapitation and organs were aseptically removed, individually homogenized and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H10 or 7H11 agar plates for *M. avium* and *M. tuberculosis*, respectively. Plates were incubated at 37ºC and *M. avium* and *M. tuberculosis* colonies were counted after 7 and 21 d, respectively.

Gene expression analysis

Total RNA was isolated from thymi, spleens and lungs using TRIZOL reagent or TRIZOL Plus RNA purification system (Invitrogen, CA, USA). Five hundred nanograms of total RNA were amplified using the Superscript RNA amplification system (Invitrogen CA, USA) according to the manufacturer's instructions. mRNA transcripts were assessed by quantitative real-time PCR (qPCR) using SsoFast™ EvaGreen Supermix® (BioRad, CA, USA) in a BioRad CFX96™ Real-Time System with a C1000™ Thermal Cycler or a Stratagene Mx3005P Thermal Cycler. The hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as reference gene. Specific oligonucleotides were used for *Hprt* (sense: 5'-GCT GGT GAA AAG GAC CTC T-3'; antisense: 5'-CAC AGG ACT AGA ACA CCT GC-3'), *Ifnγ* (sense: 5'-CAA CAG CAA GGC GAA AAA GG-3'; antisense: 5'-GGA CCA CTC GGA TGA GCT CA-3'), *Tnf* (sense: 5'-TGC CTA TGT CTC AGC CTC TTC-3'; antisense: 5'-GAG GCC ATT TGG GAA CTT CT-3'), *Cxcl9* (sense: 5'-CTT TTC CTC TTG GGC ATC AT-3'; antisense: 5'-GCA TCG TGC ATT CCT TAT CA-3'), *Cxcl10* (sense: 5'-GCT GCC GTC ATT TTC TGC-3'; antisense: 5'-TCT CAC TGG CCC GTC ATC-3'), *Ccl4* (sense: 5'-AGC ACC AAT GGG CTC TGA-3'; antisense: 5'-TTT GGT CAG GAA TAC CAC AGC -3') and inducible nitric oxide synthase (*Inos;* sense: 5'-CTC GGA GGT TCA CCT CAC TGT-3'; antisense: 5'-GCT GGA AGC CAC TGA CAC TT-3'). The cDNA was denatured for 1 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, incubation at the optimized melting temperature for 20 s, and 72 °C for 20 s. Optimized melting temperatures were 57 °C for *Cxcl9* and *Ccl4,* 58 °C for *Hprt*, *Ifnγ* and *Cxcl10* and 59 °C for *Inos*. The expression level of each gene was determined using the ΔΔCt method taking into account the efficiency of the PCR reaction (35). Data are represented as the ratio of the expression level of the gene for each infected mice over the mean expression level of the gene in uninfected mice.

Protein quantification in tissue homogenates

Protein was extracted from spleen, lung and thymus using the Bio-Plex Cell Lysis kit (Bio-Rad, CA, USA) and the concentration of IFNγ, TNF and CCL4 were measured using a mouse Bio-Plex cytokine assay (Bio-Rad, CA, USA). In the case of *M. tuberculosis*-infected tissues, the concentrations of CXCL9 and CXCL10 were measured using the mouse CXCL9/MIG Quantikine ELISA Kit or mouse CXCL10/IP-10/CRG-2 Quantikine ELISA Kit (R&D Systems, MN, USA).

Immunohistochemistry

Detection of iNOS was performed by immunohistochemistry in paraformaldehyde fixed, paraffin embedded tissues. Briefly, 5 µm thymic sections were dehydrated and antigens

were 'unmasked' by incubation at 96 °C for 30 min in 1 mM EDTA 0.05% Tween pH 8. Non-specific binding was blocked using 4% BSA in PBS with 0.05% Tween 20 and endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide for 30 min. Tissues were incubated overnight, at 4 °C, with purified rabbit anti-mouse iNOS (clone M-19, Santa Cruz Biotechnology, CA, USA) and detection was performed using a peroxidase goat anti-rabbit IgG (Vector Labs, CA, USA) followed by incubation with DAB until color development. Mycobacteria were detected by Ziehl-Neelsen, using standard procedures, after the iNOS staining. Slides were visualized using a BX61 microscope with an Olympus DP70 camera. No significant signal was observed when iNOS stain was performed in iNOS KO mice.

In vitro stimulation and IFNγ measurement by ELISA

Cell suspensions from thymus and spleen were prepared by gentle disruption of the organs between two notched slide glasses or by forcing organs through a 70 µm nylon strainer (Fisher). For lung preparations, tissue was digested for 1 h at 37 °C in 1 mg/mL collagenase (Sigma) prior to straining. Erythrocytes were lysed using a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM sodium EDTA pH 7.2) and, after washing, cells were resuspended in supplemented DMEM or RPMI (10% heat inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin, all from Invitrogen). Cells were enumerated in 4% trypan blue on a hemocytometer or on a Countess Automatic Cell Counter (Life Technologies). 5×10^5 cells were plated in each well of a 96-well plate and incubated, in triplicate, in the presence *M. avium* total extract proteins (4 µg/ml), $Ag85_{280\text{-}294}$ peptide (4 µg/ml; Metabiom, Germany) (36), $TB10.4_{4\text{-}11}$ peptide (10 µM; New England Peptide) (37) or ESAT-$6_{1\text{-}20}$ peptide (10 µM; New England Peptide) (38). Incubation in the presence of Concanavalin A (4 µg/ml; Sigma) or αCD3/αCD28 (1 mg/mL; BioLegend) or in the absence of stimuli were used as positive and negative controls, respectively. Supernatants were collected after 72 h of culture and the concentration of IFNγ was determined by ELISA (R4-6A2 and biotinylated AN18 were used as capture and detection Abs, respectively, from eBiosciences; or using the Mouse IFNγ ELISA MAX Standard kit, from Biolegend).

Flow cytometry

Surface staining was performed with antibodies specific for mouse CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD24 (M1/69), CD44 (IM7) and CXCR3 (CXCR3-173) (from Biolegend, CA, USA, or from BD Pharmingen, CA, USA). The tetramers $Ag85_{280\text{-}294}$-loaded I-A^b and $TB10.4_{4\text{–}11}$-loaded H-2 K^b were obtained from the National Institutes of Health

Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA); staining was performed for 30 min at 37 °C before incubation with the antibodies mix for the $Ag85_{280-294}$-loaded I-A^b tetramer or for 20 min on ice during the incubation with the surface antibodies for the TB10.$4_{4–11}$-loaded H-2 K^b tetramer. All stainings, except the one with the $Ag85_{280-294}$-loaded I-A^b tetramer, were fixed before acquisition with 2% formaldehyde in PBS for 30 min.

Cell analysis was performed on a LSRII flow cytometer or on a FACS Canto using FACS Diva Software (Becton Dickinson, NJ, USA). Data were analyzed using FlowJo Software (Tree Star, OR, USA). Single-lymphocyte events were gated by forward scatter versus height and side scatter for size and granularity.

Thymic transplant

Thymic lobes were aseptically removed from WT mice and kept in cold supplemented DMEM until being transplanted under the kidney capsule of anesthetized TCRα KO mice (200 µg xylazine hydrochloride and 200 µg ketamine hydrochloride, administered i.v.).

T cell chimeras

Single cell suspensions of pools of spleen and thymus from *M. avium* infected RAG-GFP mice (20 to 22 wpi) were prepared. $CD4^+$ T cells were purified from each suspension using the $CD4^+$ T cell isolation kit for spleenocytes or CD8a (Ly-2) microbeads (both from Miltenyi Biotec, Germany) for thymocytes. Magnetic separation was performed with an autoMACS separator (Miltenyi Biotec, Germany). After purification, cells were counted and stained with antibodies for CD3, CD4, CD8 and CD24 for sorting. Splenic $CD4^+CD8^-CD3^+$ cells, thymic $CD4^+CD8^-CD3^+RAG^-CD24^{lo}$ cells and thymic $CD4^+CD8^-CD3^+RAG^{int}$ cells were sorted using a FACSAria cell sorter (Becton Dickinson, NJ, USA) with a purity ranging from 98 to 100%, depending on the experiment and on the population. 4 to 4.2×10^5 cells were transferred per TCRα KO receptor mouse.

Adoptive transfer of CXCR3-expressing T cells

Single cell suspensions of pools of spleens from *M. tuberculosis* infected B6 mice (20 to 26 wpi) were prepared. Total T cells were purified from each suspension using the T cell isolation kit II (from Miltenyi Biotec, Germany). Magnetic separation was performed with an autoMACS separator (Miltenyi Biotec, Germany). After purification, cells were counted and 7×10^7 cells were transferred intravenously to each recipient. Recipient mice (CD45.1) had

been infected for 12 weeks, and were analyzed 24 h following transfer. After sacrifice, magnetic bead-based enrichment was performed on single cell suspensions of thymocytes, as previously described (39). Briefly, cells were stained with a PE-labeled antibody specific for mouse CD45.2 (104) (from Biolegend, CA, USA), followed by incubation with anti-PE beads (from Miltenyi Biotec, Germany). Magnetic separation was performed with an autoMACS separator (Miltenyi Biotec, Germany). The bound fraction, enriched for donor cells, was analyzed by FACS (as described above).

Statistical analysis

All data are represented as mean + SEM. Data were verified for Gaussian distribution or Mann-Whitney *U* test were performed to compare two groups. To compare more than 2 groups, one-way ANOVA, followed by Bonferroni post-hoc test was performed. Differences with a $p<0.05$ were considered significant and represented by *.

Results

Thymic integrity is maintained following mycobacterial infection.

The ability of mycobacteria to disseminate to the thymus has been previously described upon aerosol challenge with *M. tuberculosis* [Figure 1A and (18)] and following intravenous infection with *M. avium* (18, 19). In both cases the bacterial load in the thymus is initially low or undetectable, but increases profoundly until it plateaus, in a pattern similar to the one observed in the lung and spleen, indicative of immunological restriction of bacterial replication. However, bacterial dissemination to the thymus is delayed compared to other organs, and peaks late during the course of infection – 16 weeks post infection (wpi) for *M. avium* (18, 19) and 12 wpi for *M. tuberculosis* (Figure 1A). In addition, thymic infection by *M. tuberculosis* is also more heterogeneous, with a small fraction of mice having undetectable bacterial load within the thymus even at 24 wpi (Figure 1A). The fact that we did not observe this heterogeneity previously (18) might be a consequence of the greater number of animals studied in the present experiments or of slight differences in the infection protocol (different *M. tuberculosis* strains and/or different apparatus used for aerosol infection).

As previously observed for *M. avium* infection with a low virulence strain (19), infection with *M. tuberculosis* does not induce loss of thymic cellularity even at 24 wpi, as compared to uninfected age-matched controls (Figure 1B). Percentages of the four main thymic populations - assessed by CD3, CD4 and CD8 expression [$CD4^-CD8^-CD3^-$ double negative (DN); $CD4^+CD8^+CD3^{low/-}$ double positive (DP); $CD4^+CD8^-CD3^+$ single positive CD4 (CD4SP); $CD4^-CD8^+CD3^+$ single positive CD8 (CD8SP)] – are also maintained following infection, although a small increase in the percentages of SP cells, both CD4SP and CD8SP, and decrease in DP, is observed from 12 wpi on (Figure 1C). These data indicate that the presence of *M. tuberculosis* within the thymus does not induce premature thymic atrophy – a common consequence of systemic infection (8) – nor major alterations in thymic cell populations, as has been shown previously for *M. avium* (19).

An immune response develops in the thymus following mycobacterial infection.

The stabilization of bacterial growth in the primary infected organ, spleen for *M. avium* and lung for *M. tuberculosis,* is associated with the establishment of an effective acquired immune response (40-42). To evaluate whether an immune response is established in the thymus, the expression of key cytokines required for immunity to mycobacteria were measured in whole organ homogenates of mice infected with *M. avium* (intravenously) or with *M. tuberculosis* (by aerosol), and compared to uninfected controls.

The selected time-points reflect periods when: 1) the peak in the immune response is observed in the primary infected organs upon *M. tuberculosis* (lung) (43) and *M. avium* (spleen) infection (40) (4 wpi for both infections); 2) the bacterial burden in the thymus is still increasing (4 wpi for *M. tuberculosis*, 4 and 12 wpi for *M. avium*); 3) the bacterial burden plateaus in the thymus (12 wpi for *M. tuberculosis*, 16 wpi for *M. avium*); 4) bacterial growth in the thymus has been sustained for a long period (24 wpi) (Figure 1) (18, 19).

During both infections, IFNγ expression is significantly increased as early as 4 wpi in the primary infected organ (spleen for *M. avium* – Figure 2A; lung for *M. tuberculosis* – Figure 2B). In the thymus, IFNγ expression is increased only at later time points consistent with the delayed dissemination and establishment of bacterial control (16 wpi for *M. avium* – Figure 2A; 12 wpi for *M. tuberculosis* – Figure 2B). The kinetics of TNF expression is similar to that of IFNγ in mice infected with *M. tuberculosis* (Figure 2B). In contrast, in mice infected with *M. avium* TNF is not upregulated in the thymus and only transiently upregulated in the spleen (Figure 2A). In agreement with the observed changes in gene expression, elevated levels of IFNγ protein are detected in the thymus 16 wpi with *M. avium* (Figure 2C), and both IFNγ and TNF protein are increased in the thymus 12 wpi with *M. tuberculosis* (Figure 2D). These data show that the thymus is the site of an ongoing immune response that involves the production of pro-inflammatory cytokines after infection, and the cytokine levels peak concordantly with the control of bacterial replication.

Mycobacteria-specific T cells are detected in the thymus after infection.

To determine whether a mycobacteria-specific T cell response accompanies the inflammatory changes observed in the infected thymus, IFNγ production by thymus cells obtained from infected mice after stimulation with defined mycobacterial antigens was assessed. IFNγ production was specifically induced when spleen or thymus cells from *M. avium* infected mice were stimulated with *M. avium* protein extract or with $Ag85_{280\text{-}294}$ peptide [primarily recognized by $CD4^+$ T cells (36)]. These antigens led to maximal IFNγ production by splenocytes at 4 wpi (Figure 3A), as previously described (40). In contrast, these antigens did not trigger IFNγ production by thymic cells until 16 wpi and even then elicited much lower amounts of IFNγ (Figure 3A). Similarly, large amounts of IFNγ were produced by lung cells obtained from *M. tuberculosis*-infected mice after stimulation with the immunodominant epitope $ESAT6_{1\text{-}20}$ [recognized by $CD4^+$ T cells (38)] or $TB10.4_{4\text{-}11}$ [recognized by $CD8^+$ T cells (37)] as early as 4 wpi. Although a *M. tuberculosis*-specific response could be detected in the thymus as early as 4 wpi, a more substantial response was detected 12 wpi (Figure 3B) and, as in the case of *M. avium*, in quite lower amounts and associated with control of the infection and a plateau of the bacterial burden in the thymus (Figure 3B).

We also determined the frequency of TB10.4_{4-11}-specific $CD8^+$ T cells during *M. tuberculosis* infection using K^b/TB10.4_{4-11} tetramers, which can identify antigen-specific $CD8^+$ T cells independently of their function (Figure 3C and Supplementary Figure 1). Similar to our other data, the frequency of antigen-specific T cells closely correlated with the production of IFNγ in the infected organs. Taken together, these data indicate that mycobacteria-specific T cells are present within the thymus following infection. Although the magnitude of the response differs from that observed in the dominant target organs, the kinetics in the thymus resembles other tissue-specific responses to mycobacterial infection.

Mycobacteria-infected cells in the thymus express iNOS.

The production of IFNγ by antigen-specific T cells is a central feature of protective immunity against mycobacterial infection (44, 45). Among the important antibacterial actions of IFNγ is upregulation of inducible nitric oxide synthase (iNOS) expression by macrophages. iNOS catalyzes the production of nitric oxide (NO), which has a significant role in controlling *M. tuberculosis* infection (46). Despite the fact that NO plays no role in the protective immunity to *M. avium* (47, 48), iNOS expression represents a suitable marker of macrophage activation in this scenario. The elevated IFNγ level in the infected primary organs was associated with increased iNOS expression by 4 wpi. An increase in iNOS expression in the thymus was only observed at later time points: 24 wpi for *M. avium* (Figure 4A) and 12 wpi for *M. tuberculosis* (Figure 4B). These data are consistent with a protective T cell response in the thymus leading to iNOS induction, NO production, and control of bacterial replication.

In addition to measuring iNOS gene expression, we detected iNOS protein by immunohistochemical staining. Although iNOS is expressed in the thymus even in the absence of infection (49), we detected an increase in the number of cells containing *M. avium* (Figure 4C) or *M. tuberculosis* (Figure 4D) and expressing iNOS throughout the course of infection, with most of the infected cells expressing iNOS at later time points. For *M. avium* and *M. tuberculosis*, most infected cells in the thymus were located in the medulla or corticomedullary region, although occasional bacteria were observed in the cortex. *M. tuberculosis*-infected cells were also infrequently observed in the subcapsular zone. Thus, our data showing augmented iNOS expression and its colocalization with infected cells indicates the establishment of a bona fide protective immune response within the thymus.

Mycobacteria-infected thymi contain cells able to transfer protection against infection.

Having shown that the thymus is a site of infection where antigen-specific immune responses are detected, we next asked whether cells within infected thymi could confer

protection against subsequent infection. To that end, we transplanted *M. avium*-infected or uninfected thymic lobes from WT mice under the kidney capsule of TCRα KO mice (33), that lack peripheral $\alpha\beta$ T cells (Figure 5A).

The transplanted mice were challenged with *M. avium* and sacrificed at 4 or 8 wpi, at which time thymic engraftment was confirmed macroscopically. At both time points, the spleens of mice transplanted with infected or uninfected thymic lobes had similar numbers of $CD4^+$ T cells, indicating that cells leaving the infected or uninfected thymus were equally able to reconstitute the peripheral T cell pool of the recipient TCRα KO mice (Figure 5B). Both groups receiving thymic transplants had lower bacterial burdens than the untransplanted TCRα KO mice (Figure 5C). This demonstrates that the cells emerging from the thymic grafts are functional and can mediate protection against microbial pathogens. Importantly, mice receiving infected thymic grafts were significantly more protected than those receiving uninfected grafts, despite similar T cell reconstitution (Figure 5C). These data support the hypothesis that the greater protection conferred by the infected thymic grafts is mediated by *M. avium*-specific T cells contained within the grafts, that efficiently confer protection against infectious challenge.

Mycobacteria-specific T cells within the thymus are recirculating cells.

After establishing that antigen-specific T cells within the infected thymus produce protective cytokines, induce NO production, and transfer protection, we next sought to determine the origin of these cells. In particular, we wished to determine whether differentiating T cells were primed in the thymus, or alternately, whether mature mycobacteria-specific T cells, primed in the periphery, traffic to the thymus.

Our previous data demonstrated that T cells that differentiate within *M. avium*-infected thymi are tolerant to the invading pathogen (19). Moreover, newly differentiated T cells arising from the thymus are not fully mature (50) and need additional signals within secondary lymphoid organs to achieve full differentiation (51, 52). Therefore, we used a genetic model in which GFP is expressed under the control of the RAG2 promoter (34), for the purpose of identifying newly differentiated T cells in the thymus. The RAG2 enzyme is essential for genetic recombination of B and T cell antigen receptors and is down-regulated afterwards. Even though RAG2 is down-regulated after T cell differentiation in the thymus, cells remain GFP^+ for about 2 weeks (50). As such, it is highly expressed in differentiating T cells and its expression is a useful marker to distinguish between newly differentiated T cells (high to intermediate GFP expression) and mature peripheral T cells (no GFP expression).

When RAG-GFP mice were infected with *M. avium* there was no difference on the percentage of GFP^- among CD4SP cells, suggesting that there is no difference on the

amount of thymic recirculating T cells (Figure 6A and Supplementary Figure 1). However, upon infection most of the $Ag85_{280-294}$-specific CD4SP cells in the thymus were GFP^-, showing a modification in the repertoire of recirculating T cells (Figure 6A). In contrast with what was observed for *M. avium*, thymi from *M. tuberculosis* infected mice showed an increase in the percentage of GFP^- within CD8SP cells, suggesting an increase in T cell trafficking from the periphery back to the thymus (Figure 6B). A similar increase was detected when analyzing mycobacteria-specific T cells ($TB10.4_{4-11}$-specific CD8SP T cells) during *M. tuberculosis* infection, in agreement with what was observed for antigen-specific cells in the thymus of *M. avium* infected mice (Figure 6B). In both cases, no tetramer positive cells were found in the thymus of control uninfected mice, which is in agreement with the small precursor frequency we observe for T cells reactive against these mycobacterial antigens in naïve mice (data not shown). In parallel, we show that the majority of $TB10.4_{4-11}$-specific CD8SP T cells within the thymus of WT mice infected with *M. tuberculosis* are $CD44^{hi}CD24^{lo}$ (Figure 6C), a phenotype typical of re-circulating $CD8^+$ T cells (25, 53).

T cell chemokines are increased in the thymus during infection.

The above results indicate that antigen-specific T cells from the periphery respond to infection in the thymus by trafficking back to this organ. To determine whether increased chemokine production is associated with the recruitment of mycobacteria-specific T cells to the thymus, we measured the expression of chemokines that recruit T_{h1} cells (54), including CXCL9, CXCL10 and CCL4, after *M. avium* and *M. tuberculosis* infection. We found increased mRNA expression of all three chemokines in the primary infected organs during *M. tuberculosis* infection and at least at the initial phase of *M. avium* infection (Figure 7A and B). These chemokines were also detected in the thymus though at lower levels and at later time points – after 16 wpi for *M. avium* (Figure 7A) and 12 wpi for *M. tuberculosis* (Figure 7B). At the protein level, increased CCL4 was detected in the spleen but not in the thymus during *M. avium* infection, possibly because the expression levels were low (data not shown). During *M. tuberculosis* infection, the expression of these chemokines was strongly induced in the lungs of mice infected for 4 weeks, and the responses appeared maximal for all three chemokines by 12 wpi (Figure 7C). These chemokines were also highly expressed in the thymus, but their expression was not detected until 12 wpi (Figure 7C).

The high levels of CXCL9 and CXCL10 detected in the infected thymus led us to investigate whether mycobacteria-specific T cells express CXCR3, the receptor for these chemokines (54). We found the majority of $TB10.4_{4-11}$-specific CD8SP T cells in *M. tuberculosis* infected thymi to express high levels of the chemokine receptor CXCR3 (Figure 7D and E).

To support a role for CXCR3 in the recruitment of antigen-specific T cells to the infected thymus, we adoptively transferred purified splenic T cells obtained from *M. tuberculosis* infected donors into congenically marked infected recipients (Supplementary Figure 2). Analysis of the donor cells immediately before transfer found that 30-40% of the $CD4^+$ and $CD8^+$ T cells expressed CXCR3. 24 hrs after injection, the proportion of donor $CD4^+$ and $CD8^+$ donor T cells recruited to the thymus that expressed CXCR3 was increased to 65-85%. The increased CXCR3 expression was limited to the infected thymus, and was not observed in the spleen or the lung of recipient mice (Figure 7F and Supplementary Figure 2). This data suggests that CXCR3 participates in the recruitment of antigen-specific cells to the infected thymus. Thus, although the thymus is the key organ for T cell differentiation, mycobacteria-specific T cells from the periphery are recruited to the thymus following infection. Our data is consistent with the chemokines CXCL9 and CXCL10 playing an important role in the recruitment of these antigen-specific T cells from peripheral organs to the thymus in order to fight infection.

Peripheral recirculating T cells within infected thymi efficiently confer protection against infection.

To confirm that peripheral T cells recirculating to infected thymi are the ones that confer protection to infection, RAG-GFP mice were infected with *M. avium* and 20 wpi sacrificed and their thymi and spleens collected. Highly purified T cell populations were sorted accordingly to the expression of GFP and of surface markers: 1) total $CD4^+$ T cells from the spleen ($CD3^+CD4^+CD8^-$); 2) thymic recirculating CD4SP cells ($CD3^+CD4^+CD8^-GFP^-CD24^{lo}$); 3) thymic newly differentiated CD4SP cells ($CD3^+CD4^+CD8^-GFP^{int}$). A GFP^{int} population was sorted to the detriment of the total GFP^+ population as we wished to exclude the most immature GFP^{hi} CD4SP cells (Figure 8A). Each cell subset was transferred to TCRα KO mice that had been infected the previous day with *M. avium*. Eight weeks later, the mice were analyzed (Figure 8B).

Despite receiving the same number of cells, mice that received recirculating thymic CD4SP cells (GFP^-) had lower numbers of $CD4^+$ T cells than animals that received either newly differentiated T cells from the thymus (GFP^{int}) or total splenic T cells (Figure 8C, left panel). Nevertheless, administration of recirculating thymic CD4SP cells provided significantly more protection from *M. avium* infection in the spleen and liver compared to animals that received newly differentiated thymic T cells (Figure 8C, middle and right panels). In addition, the protection provided by recirculating thymic CD4SP T cells is comparable to that of total splenic $CD4^+$ T cells. Although transfer of newly differentiated T cells from the thymus provided partial protection against *M. avium* infection in comparison to TCRα KO

mice, this was significantly reduced compared to protection conferred by recirculating thymic CD4SP cells. Taken together, these results indicate that T cells recirculating from the periphery back into the thymus are capable of protecting mice against infection.

Discussion

Our understanding of the role of T cells in resistance to microbial infection is generally limited to the effector or memory phases of the immune response, or their role in vaccine-induced immunity. Despite the key role of the thymus in generating such T cells, the consequences of infection of the thymus itself have been rarely studied. Our previous studies have shown that mycobacteria disseminate to the thymus (18, 19) and alter the process of T cell differentiation leading to tolerance against the invading pathogen (19). Here, we address whether and how the immune system is able to defend the thymus against infection, to preserve its structure, and its ability to generate T cells.

Our results show that like other tissues that are the target of mycobacterial infection, mycobacteria-specific T cells appear in the thymus following the dissemination and subsequent growth of bacteria. Furthermore, the appearance of antigen-specific T cells roughly correlates with the host's ability to control bacterial growth in the thymus. Antigen-specific T cells in the thymus secrete IFNγ and stimulate antimicrobial functions of infected cells, as manifested by the upregulation of iNOS. Thus, T cell responses in the thymus resemble ones occurring in the lung or spleen although they differ in magnitude. In fact, both the number of antigen-specific T cells and related immune molecules detected in the thymus is significantly lower than found in the lung or the spleen. Although the thymic immune response is less pronounced, its ability to control bacterial growth seems similarly efficient as the one established in other peripheral organs. Additionally, our previous data shows that cellular infiltrates and granuloma-like lesions, characteristic of inflammation in other tissues, are not detected in the infected thymus (18). The reasons for this difference are not clear, but we speculate that avoiding a massive pro-inflammatory state and preventing disruption of the cellular architecture is required for the thymus to remain functional. The smaller magnitude of the thymic-associated immune response might contribute to the maintenance of thymic integrity during the course of infection. It has previously been shown that infection with a highly virulent *M. avium* strain, which is associated with a vigorous immune response, causes robust thymic atrophy (48). Recent results from our laboratory (55) show that this depends on a synergistic effect between IFNγ-induced iNOS induction and corticosteroid production, as blocking the effect of each one individually prevents thymic atrophy. Of notice, infection with the low virulence *M. avium* strain used in the present work is not accompanied by increased serum levels of corticosteroids (55), which may contribute to the maintenance of thymic integrity reported here.

An important question that our data address is the origin of antigen-specific T cells in the thymus that mediate protection against mycobacterial infection. In our model, T cells from the periphery recirculate back to the thymus and are responsible for bacterial control.

Thus, although the thymus maintains the differentiation of T cells during infection, these do not appear to be the cells that defend the thymus against infection. These data are consistent with our previous results showing that newly differentiated T cells that mature in a thymus infected by *M. avium* are tolerant to mycobacterial antigens, and consequently are not optimally able to establish protective immunity(19). Interestingly, T cells that differentiate in an infected thymus were not completely impaired in their ability to proliferate, to traffic to infected organs, and to confer a small amount of protection against infection, albeit less efficiently than T cells that develop within an uninfected thymus (19). In agreement with those findings, we now report that newly differentiated T cells are much less effective at conferring protection than peripheral T cells that recirculate back to thymus. Therefore, we conclude that recirculation of peripheral activated T cells back to the thymus is a mechanism to survey and protect this organ from invading pathogens.

These data are also in agreement with other work that finds newly differentiated T cells defective in T_{h1} commitment (dampened cytokine production and transcriptional factor expression) and biased toward the T_{h2} lineage (56), and a requirement for newly differentiated T cells to exit the thymus and reside in the periphery before they become fully functional (50-52).

Another interesting question arising from this study concerns whether the antigen-specific cells found in the thymus are primed in peripheral organs and traffic back to the thymus because of thymic infection, or are recirculating T cells that traffic back and are then primed within the infected thymus. Although we have not addressed this question directly, our data on the production of T_{h1} chemokines within the infected thymus, the expression of the cognate chemokine receptors by the antigen-specific T cells found within this organ, and the preferential recruitment of $CXCR3^+$ cells to the infected thymus, strongly supports the notion that the antigen-specific T cells found in the thymus are primed in other tissues during infection and then traffic to the thymus in response to a chemokine gradient.

Thymic recirculating T cells, the focus of multiple studies, have had numerous functions attributed to them including thymic surveillance, tolerance induction, and even modulation of both negative and positive selection (25-27). To our knowledge, the results presented here are the first evidence that recirculating T cells fight infection in the thymus, a function that has been previously hypothesized (25, 57, 58). The observation that mycobacteria-specific T cell trafficking to the thymus is associated with an increase in the levels of T_{h1}-related chemokines in this organ, and the expression of specific chemokine receptors such as CXCR3 by the recirculating T cells, lead us to propose that the immune system has evolved mechanisms to recruit peripheral T cells to the thymus during infection. This might ensure that invading pathogens are unable to disrupt the thymus, the primary lymphoid organ where T cells are generated. Such mechanisms must be particularly

relevant for pre-natal and early childhood infections. In both scenarios, pathogen dissemination to the thymus could lead to the presentation of microbial antigens in a “self” context, and lead to the deletion of developing T cells that recognize pathogen-specific antigens. Exclusion of pathogen reactive T cells from the peripheral pool could have severe effects during childhood, when the peripheral T cell pool is still developing, but the consequences could persist throughout adulthood.

Childhood infections are a great cause of morbidity and mortality, particularly in the developing world (59). Interestingly, HIV and *M. tuberculosis*, two of the most important pathogens in infants and young children, are both able to infect the thymus (16, 60) and thymic infection alters the output and selection of T cells in experimental models using these pathogens (9, 19). The evidence that thymic infection can compromise thymic function implies that the recruitment of protective immune responses to the thymus is essential to prevent detrimental alterations in immunological function.

The implications of our study extend beyond childhood infection. Skepticism about the potential immunological consequences of thymic infection would not be surprising if one were to believe the dogma the adult thymus becomes immunologically useless as it ages and progressively loses function. However, our understanding of the role of the thymus during adulthood is rapidly evolving. Interestingly, the control of persistent infections has been suggested to benefit from the continuous recruitment of naïve T cells generated in the thymus (61, 62). In this case, alterations in the development of these T cells could be detrimental to the ongoing immune response. The integrity of the adult thymus is required for other aspects of ongoing immune responses such as antibody generation (63). This observation raises the hypothesis that thymic disruption secondary to infection can impair T cell responses to infection as well as other components of the immune system. Additionally, decreased thymic output contributes to the progression from HIV infection to AIDS (7, 64), indicating that thymic export is important in the context of chronic infection in adults, especially in the context of *M. tuberculosis*/HIV co-infection. Combined, these data show that thymic infection has an impact on the development of the immune system and ongoing immune responses to pathogens. Thus, it is not surprising that mechanisms have evolved to specifically respond to the invasion of the thymus by microbial pathogens. We propose that the recirculation of T cells from the periphery to the thymus, and the presence of mature T cells in this organ, represent strategies that evolved to protect the thymus and sustain its activity during and after infectious episodes.

T cells home to the thymus and control infection

Disclosures

The authors have declared that no conflict of interest exists.

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Footnotes

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[2] Both authors contributed equally to this work.

[3] Both senior authors contributed equally to this work.

[4] Abbreviations used in this manuscript: BCG, bacillus Calmette-Guérin; DN, double negative; DP, double positive; iNOS, inducible NO synthase; SP, single positive; WPI, weeks post infection; WT, wild-type.

Figure Legends

Figure 1. *M. tuberculosis* infection of the thymus. A. Bacterial burden in the lung, spleen and thymus after aerosol infection with *M. tuberculosis*. Data is pooled from eleven independent experiments each with 3 to 7 subjects per time point, for a total 97 subjects. Each dot represents one mouse, the solid line is the mean, and the dashed line is the lower detection limit. Number of thymocytes (B) and percentage of the main thymocyte populations (based on CD3, CD4 and CD8 expression) (C) after *M. tuberculosis* infection. Both infected (closed bars) and age-matched uninfected controls (open bars) are represented. *, $p<0.05$ by Mann-Whitney *U* test. Bars represent the mean ± SEM (n = 5 mice/group). Data is representative of two independent experiments.

Figure 2. An immune response is established in the thymus after mycobacteria infection. RNA expression levels were determined by qPCR (A, B) and protein concentration were determined by multiplex (C, D) in tissues of *M. avium* (A, C) and *M. tuberculosis* (B, D) infected mice. Bars refer to fold increase of infected mice in comparison to the average of uninfected mice and represent mean ± SEM (n = 4 to 8 mice/group). Data is representative of 2 to 3 independent experiments. *, $p<0.05$ by Mann-Whitney *U* test (statistics were performed by comparing uninfected with infected mice, before performing the ratio).

Figure 3. Mycobacteria-specific T cell responses are detected in the thymus after *M. avium* and *M. tuberculosis* infection. Cells from *M. avium* (A) or *M. tuberculosis* (B) infected mice were stimulated in vitro in the presence of *M. avium* protein extract or $Ag85_{280-294}$ peptide (A) and $ESAT6_{1-20}$ or $TB10.4_{4-11}$ peptides (B). Age-matched uninfected mice were used as controls. Unstimulated and Concanavalin A or αCD3/αCD28 stimulated cultures were used as negative and positive controls of the in vitro stimulation, respectively (data not shown). IFNγ quantification in cell supernatants was performed by ELISA. C. TB10.4-specific $CD8^+$ T cells were detected in lung and thymus of *M. tuberculosis* infected mice using the $K^b/TB10.4_{4-11}$ tetramer. Closed symbols represent infected mice and open symbols uninfected mice. Data points represent the mean ± SEM (n = 4 to 6 mice/group). Data is representative of two to four independent experiments. *, $p<0.05$ by Mann-Whitney *U* test.

Figure 4. Mycobacterial infection in the thymus is associated with increased iNOS expression. iNOS RNA expression levels were quantified by qPCR in tissues of *M. avium* (A) and *M. tuberculosis* (B) infected mice. Bars refer to fold increase of infected mice in comparison to the average of uninfected mice and represents the mean ± SEM (n=4 to 8 mice/group). Data is representative of 2 to 3 independent experiments. *, $p<0.05$ by Mann-Whitney *U* test (statistics were performed by comparing uninfected with infected mice, before performing the ratio). C, D. Representative medullary thymic sections of *M. avium* (C) and *M. tuberculosis* (D) infected thymi stained for iNOS (brown). Bacilli were detected by Ziehl-Neelsen staining. Shown are representative images obtained from the analysis of 3 to 5 thymi per time-point from of 2 to 3 independent experiments. Bar = 10µm.

Figure 5. *M. avium*-infected thymi contain T cells able to confer protection during infection. A. Schematic representation of the experiment. Thymic lobes from *M. avium* infected mice (24 wpi) or from uninfected WT mice were transplanted under the kidney capsule of TCRα KO receptor mice. Transplanted mice were infected 2 to 3 days post-transplant and sacrificed 4 and 8 weeks later. Non-transplanted TCRα KO mice were used as controls. Number of $CD4^+$ T cells (B) and bacterial load (C) were assessed in the spleen. Each column represents mean ± SEM (n = 4 mice/group) from one of two experiments. *, $p<0.05$ by one-way ANOVA.

Figure 6. Mycobacteria-specific T cells recirculate to the thymus upon infection. A. Total recirculating CD4SP cells from the thymi of naïve or *M. avium*-infected RAG-GFP mice and recirculating CD4SP Ag85-specific cells from the thymi of infected mice were enumerated. Represented are density plots of the expression of the GFP marker in total CD4SP cells (left) and in CD4SP $Ag85^+$ cells (right). B. Total recirculating CD8SP cells from the thymi of naïve or *M. tuberculosis*-infected RAG-GFP mice and recirculating CD8SP TB10.4-specific cells from the thymi of infected mice were enumerated. Represented are density plots of the expression of the GFP marker in total CD8SP cells (left) and in CD8SP $TB10.4^+$ cells (right). C. In WT mice, using the surface markers CD44 and CD24, total recirculating CD8SP cells and recirculating CD8SP TB10.4-specific cells from the thymi of infected mice were enumerated. Represented are density plots of the expression of CD44 and CD24 in total CD8SP cells (left) and in CD8SP $TB10.4^+$ cells (right). All represented dot plots show concatenated data from all animals of the group. Bars represent mean ± SEM. Data is pooled from 2 to 3 independent experiments, with 5 to 8 mice/group. *, $p<0.05$ by one-way ANOVA.

Figure 7. Recirculation of mycobacteria-specific T cells into infected thymi correlates with increased Th1 recruiting chemokines and their cognate receptors. RNA expression (A, B) and protein concentration (C) were determined in tissues of *M. avium* (A) and *M. tuberculosis* (B, C) infected mice. Bars refer to fold increase of infected mice in comparison to the average of uninfected mice and represents mean ± SEM (n = 4 to 8 mice/group). Data is representative of 2 to 3 independent experiments. *, $p<0.05$ by Mann-Whitney *U* test (statistics were performed by comparing uninfected with infected mice, before performing the ratio). D. FACS plot of CXCR3 expression in thymic CD8SP TB10.4$^-$ and CD8SP TB10.4$^+$ cells from *M. tuberculosis*-infected mice. Represented is the concatenated data from 3 mice analyzed. Gating strategy is depicted in Supplementary figure 1. E. Percentage of CXCR3 expressing cells between CD8SP TB10.4$^-$ and CD8SP TB10.4$^+$ cells in *M. tuberculosis*-infected mice. Each bar represents mean ± SEM (n = 4 to 8 mice/group). Data is representative of 2 to 3 independent experiments. F. CXCR3 expression by donor CD4SP and CD8SP in the spleen and thymus. T cells from *M. tuberculosis*-infected CD45.2 mice were transferred to *M. tuberculosis*-infected CD45.1 recipient mice, and their CXCR3 expression was analyzed the next day. See Supplementary Figure 2 for scheme and gating strategy. Each bar represents mean ± SEM (n = 3 mice). Data is representative of 3 independent experiments. *, $p<0.05$ by one-way ANOVA.

Figure 8. Thymic recirculating T cells from *M. avium*-infected mice efficiently confer protection to infection. 20 weeks after *M. avium* infection, RAG-GFP mice were sacrificed and their thymi and spleens removed. Spleen CD4$^+$CD3$^+$ cells, thymic CD3$^+$CD4$^+$CD8$^-$GFP$^-$ CD24^{lo} (recirculating) cells and thymus CD3$^+$CD4$^+$CD8$^-$GFP^{int} cells were sorted and 4 to 4.2×10^5 cells (depending on the experiment) were transferred to TCRα KO mice one day after *M. avium* infection. Mice were sacrificed 8 weeks post-transfer. Non-transplanted TCRα KO mice were used as controls. A. Purity of the transfered thymic populations. Thymic cell suspensions were depleted of CD8$^+$ cells (top panels); and CD3$^+$CD4$^+$CD8$^-$GFP^{int} (middle panels) and CD3$^+$CD4$^+$CD8$^-$GFP$^-$CD24^{lo} (bottom panels) cells populations were sorted. B. Schematic representation of the experiment. C. Number of spleen CD4$^+$ T cells (left panel) and bacterial load in the spleen (middle panel) and liver (right panel) were assessed. Each column represents mean ± SEM (n = 4 to 9 mice/group) from two combined independent experiments. *, $p<0.05$ by one-way ANOVA.

Supplemental Figure 1. Gating strategy showing the analysis of (A) *M. avium* or (B) *M. tuberculosis*-specific T cells located the thymus. Single cell suspensions from thymus were gated for by forward and side scatter and then analyzed for their expression of CD4 and CD8. CD3-expressing (A) CD4SP or (B) CD8SP were analyzed using $Ag85_{280-294}$-loaded I-A^b and $TB10.4_{4–11}$-loaded H-2 K^b tetramers. Dual tetramer staining was used to identify the $TB10.4_{4–11}$-specific CD8+ T cells in *M. tuberculosis* infected mice. Thymi from age-matched uninfected and infected mice were analyzed in parallel to assist with defining the positive gates.

Supplemental Figure 2. Adoptive transfer of CXCR3-expressing T cells. (A) Experimental strategy to analyze the role of CXCR3 in the trafficking of *M. tuberculosis*–specific T cells to the thymus. (B) Gating strategy for the phenotypic analysis of donor cells in the tissues of recipient mice. Single cell suspensions were obtained from different tissues and lymphocytes gated based on FSC and SSC. Donor T cells were detected by their expression of CD3 and CD45.2. CD4+ and CD8+ T cells were identified and their expression of CXCR3 was determined. All represented dot plots show concatenated data from all animals of the group. (C) Expression of CXCR3 by donor CD4 (top bar graph) and CD8 T cells (bottom bar graph). CXCR3 expression by the donor T cells before transfer (first bar in each set) and after transfer in the different tissues of the *M. tuberculosis* infected mice. The donor CD4+ and CD8+ T cells found in the spleen and lung are not enriched in CXCR3 expression compared to the transferred T cells. In contrast, the donor T cells found in the thymus are significantly enriched for CD4+ and CD8+ T cells that express CXCR3. Each column represents mean ± SEM (n = 3 independent experiments with 3-5 recipient mice per experiment). Lines represent differences that were statistically significant *, $p<0.05$ by one-way ANOVA.

Figure 1

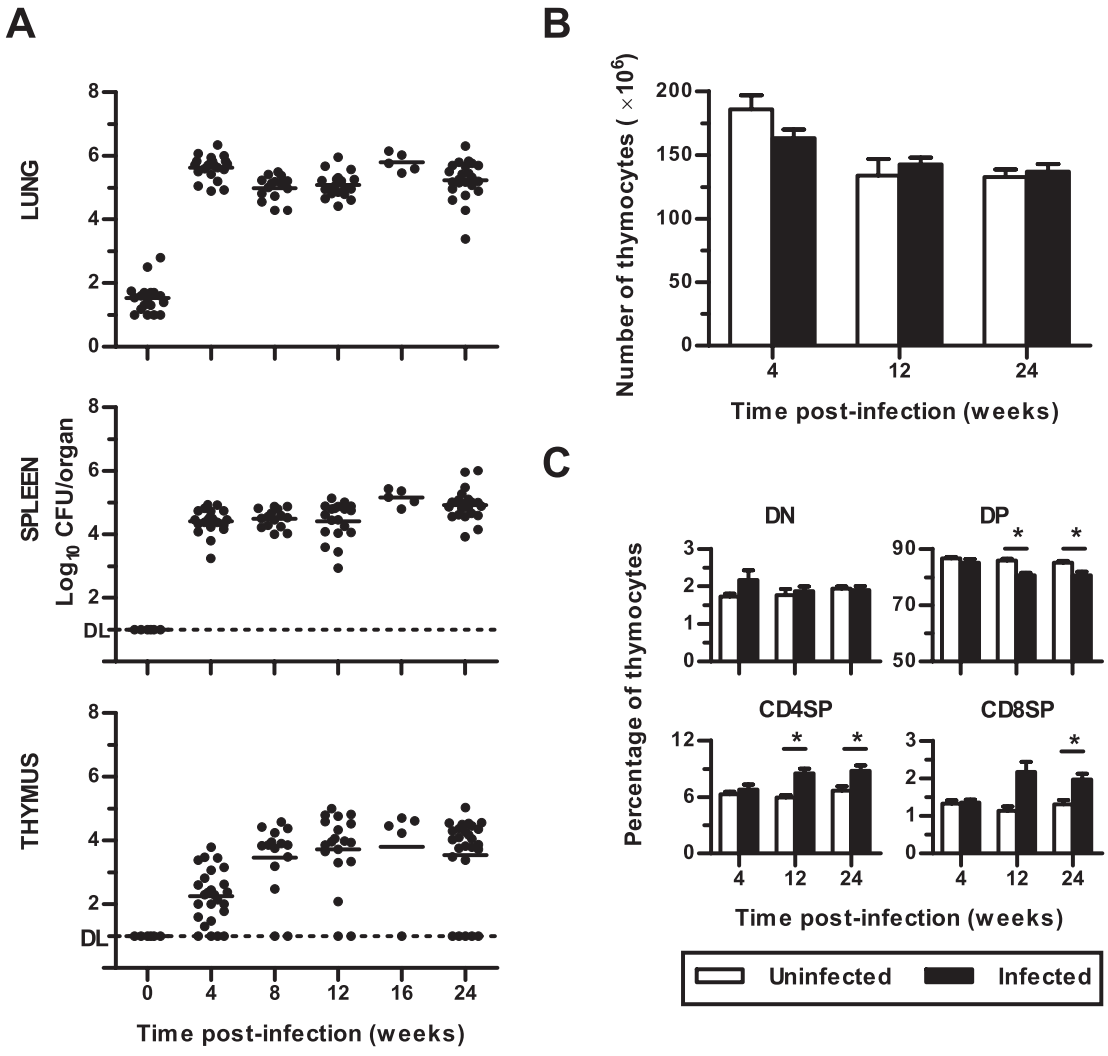

A
LUNG
SPLEEN
THYMUS
Log10 CFU/organ
DL
0
2
4
6
8
0
4
8
12
16
24
Time post-infection (weeks)
B
Number of thymocytes (×10^6)
0
50
100
150
200
4
12
24
Time post-infection (weeks)
C
Percentage of thymocytes
DN
DP
CD4SP
CD8SP
*
Time post-infection (weeks)
Uninfected
Infected

Figure 2

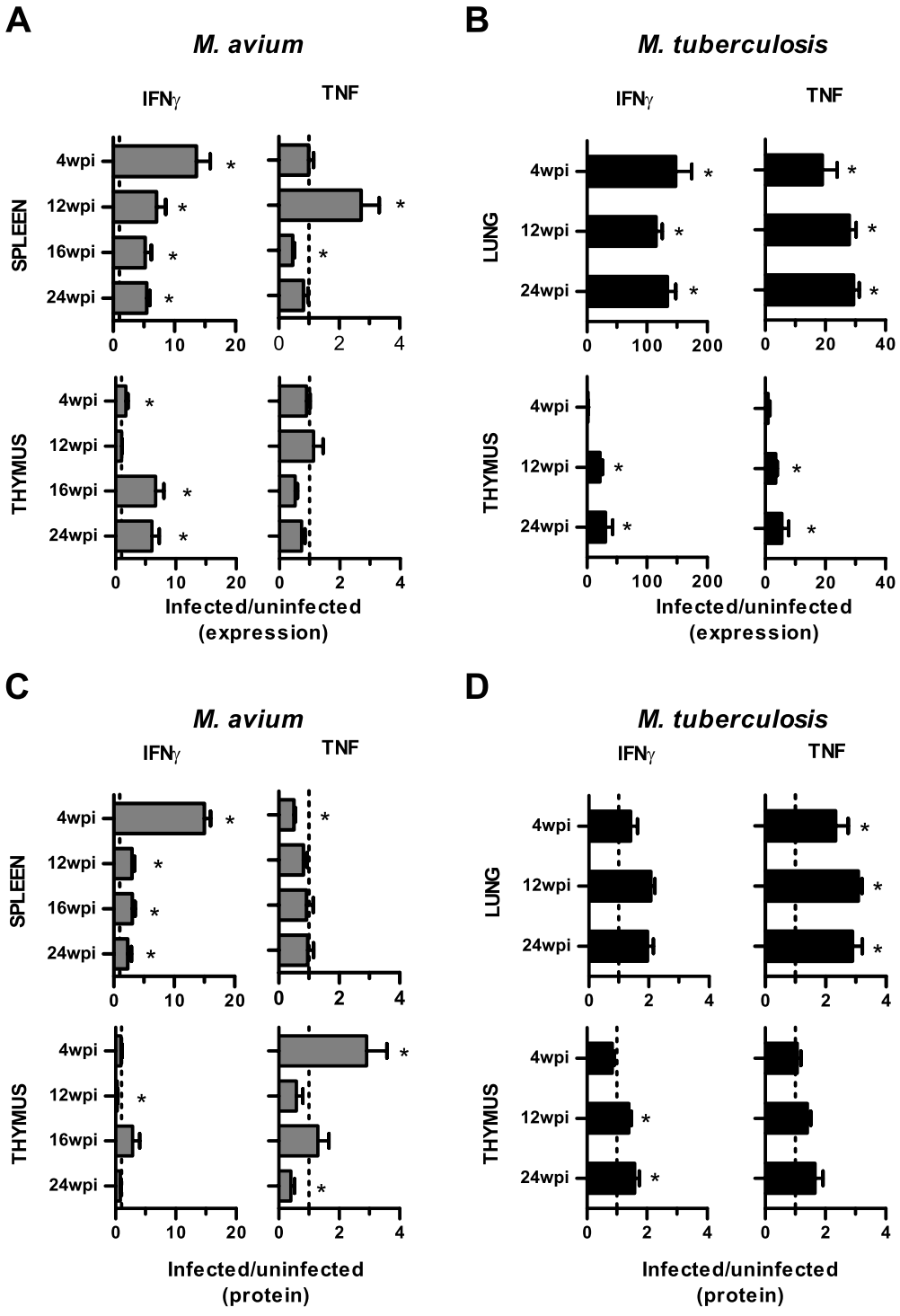

A
M. avium
IFNγ
TNF
SPLEEN
THYMUS
4wpi
12wpi
16wpi
24wpi
0
10
20
0
2
4
Infected/uninfected
(expression)
B
M. tuberculosis
IFNγ
TNF
LUNG
THYMUS
4wpi
12wpi
24wpi
0
100
200
0
20
40
Infected/uninfected
(expression)
C
M. avium
IFNγ
TNF
SPLEEN
THYMUS
4wpi
12wpi
16wpi
24wpi
0
10
20
0
2
4
Infected/uninfected
(protein)
D
M. tuberculosis
IFNγ
TNF
LUNG
THYMUS
4wpi
12wpi
24wpi
0
2
4
Infected/uninfected
(protein)

Figure 3

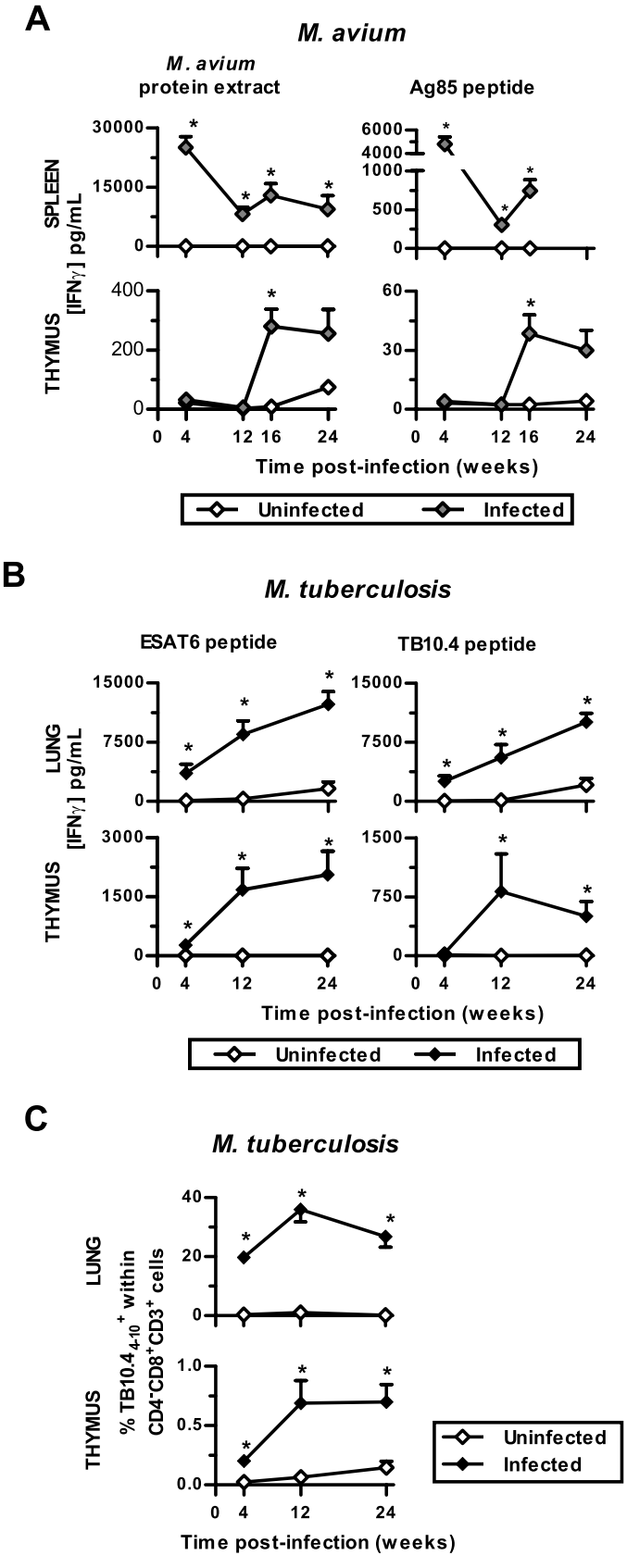

A
M. avium
M. avium protein extract
Ag85 peptide
SPLEEN [IFNγ] pg/mL
THYMUS [IFNγ] pg/mL
30000
15000
0
6000
4000
1000
500
400
200
60
30
0 4 12 16 24
Time post-infection (weeks)
Uninfected
Infected
B
M. tuberculosis
ESAT6 peptide
TB10.4 peptide
LUNG [IFNγ] pg/mL
THYMUS [IFNγ] pg/mL
15000
7500
3000
1500
750
0 4 12 24
Time post-infection (weeks)
Uninfected
Infected
C
M. tuberculosis
LUNG
THYMUS
% TB10.4 4-10 + within CD4-CD8+CD3+ cells
40
20
1.0
0.5
0.0
0 4 12 24
Time post-infection (weeks)
Uninfected
Infected

Figure 4

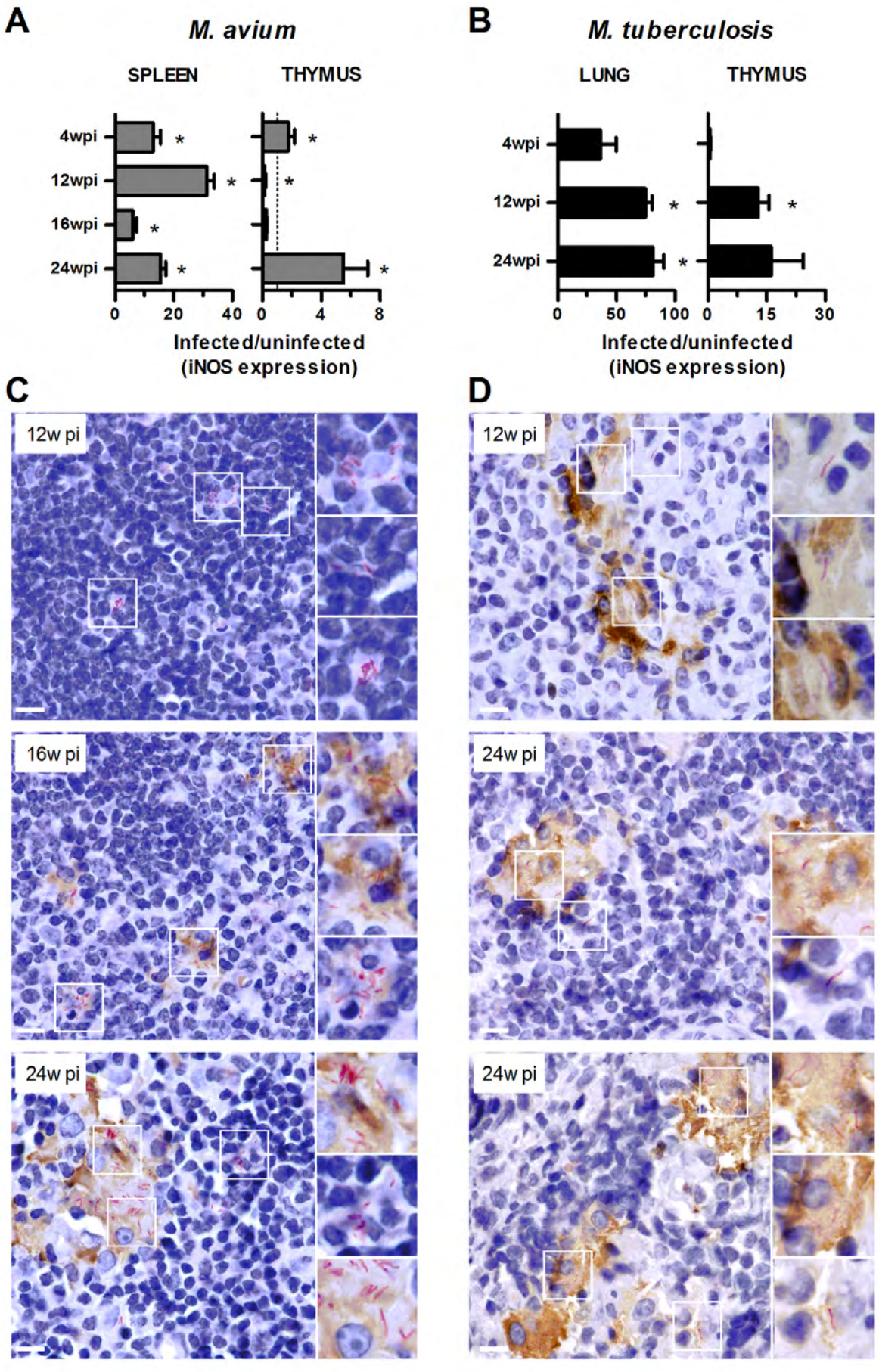

A
M. avium
SPLEEN
THYMUS
4wpi
12wpi
16wpi
24wpi
0
20
40
0
4
8
Infected/uninfected
(iNOS expression)
B
M. tuberculosis
LUNG
THYMUS
4wpi
12wpi
24wpi
0
50
100
0
15
30
Infected/uninfected
(iNOS expression)
C
12w pi
16w pi
24w pi
D
12w pi
24w pi
24w pi

Figure 5

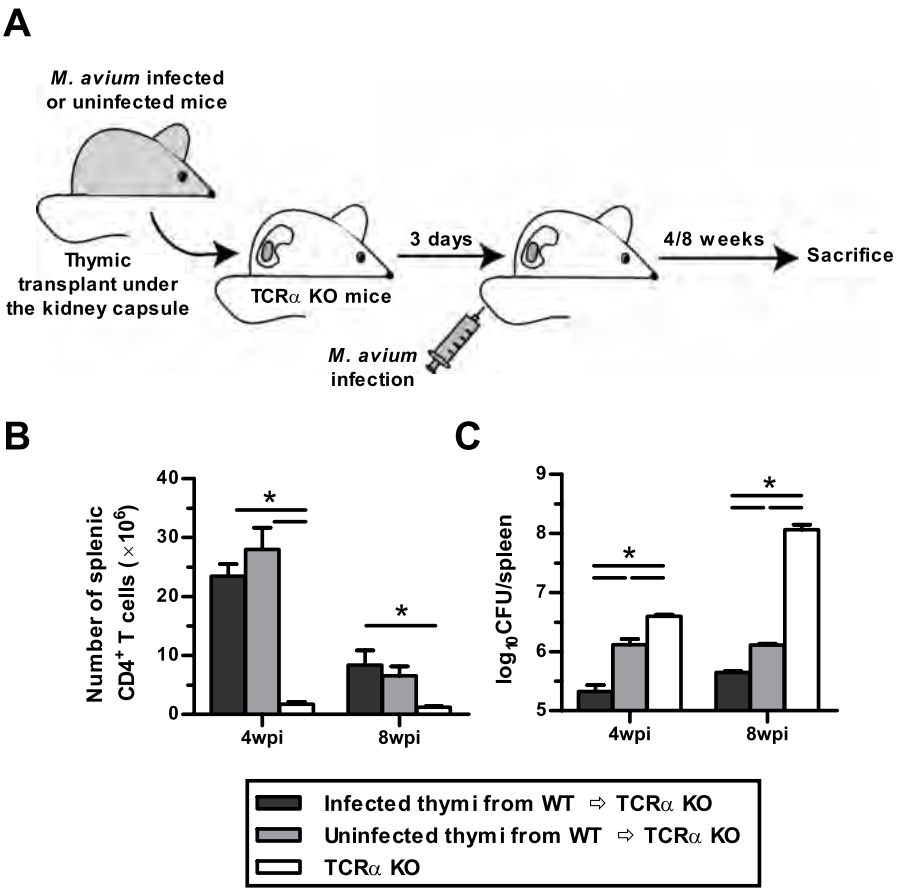

A
M. avium infected
or uninfected mice
Thymic
transplant under
the kidney capsule
TCRα KO mice
3 days
4/8 weeks
Sacrifice
M. avium
infection
B
Number of splenic
CD4+ T cells (×10^6)
40
30
20
10
0
*
*
4wpi
8wpi
C
log10CFU/spleen
9
8
7
6
5
*
*
4wpi
8wpi
Infected thymi from WT ⇨ TCRα KO
Uninfected thymi from WT ⇨ TCRα KO
TCRα KO

Figure 6

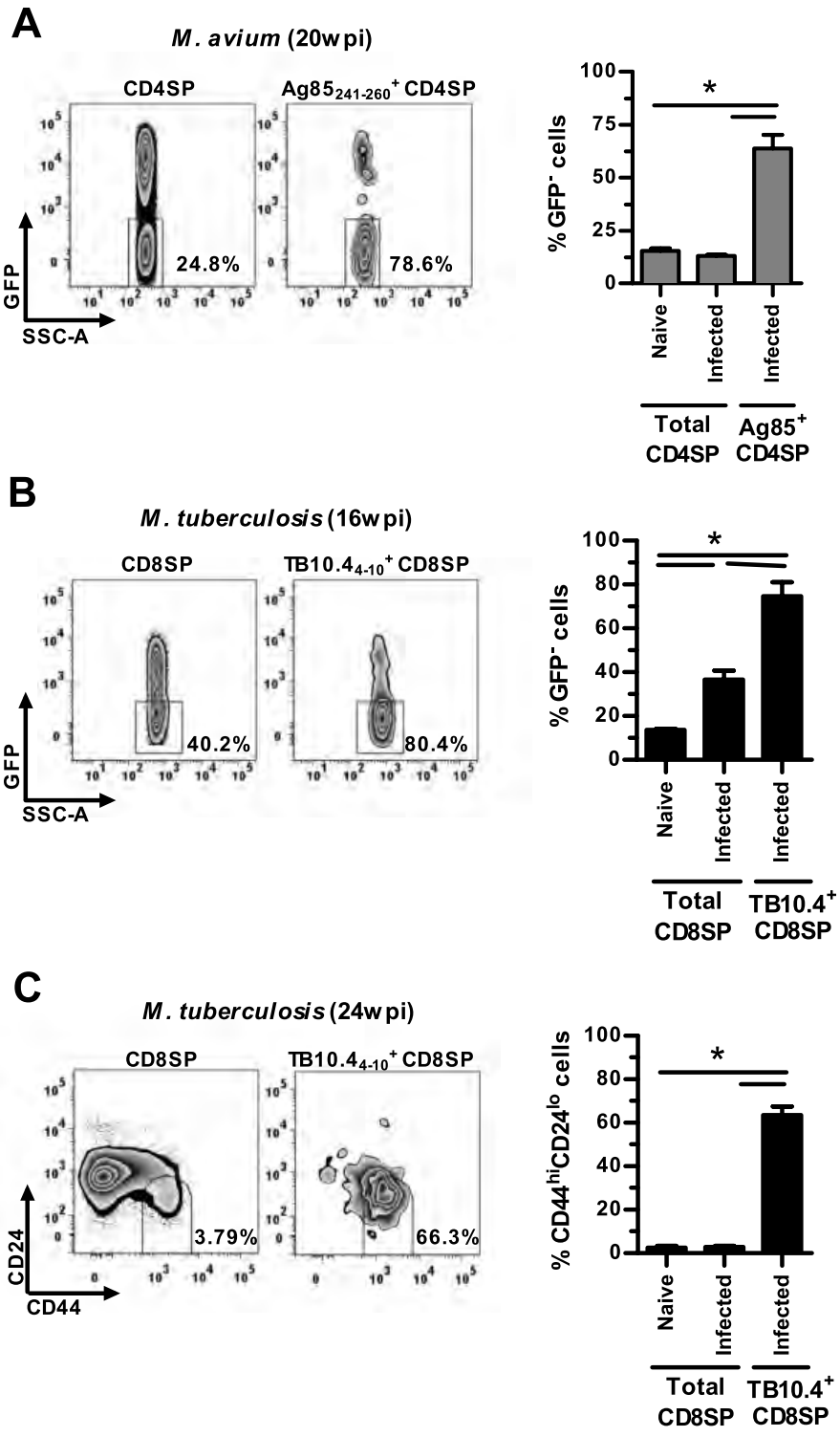

A
M. avium (20w pi)
CD4SP
Ag85241-260+ CD4SP
24.8%
78.6%
GFP
SSC-A
% GFP- cells
Naive
Infected
Infected
Total CD4SP
Ag85+ CD4SP
B
M. tuberculosis (16w pi)
CD8SP
TB10.44-10+ CD8SP
40.2%
80.4%
GFP
SSC-A
% GFP- cells
Naive
Infected
Infected
Total CD8SP
TB10.4+ CD8SP
C
M. tuberculosis (24w pi)
CD8SP
TB10.44-10+ CD8SP
3.79%
66.3%
CD24
CD44
% CD44hiCD24lo cells
Naive
Infected
Infected
Total CD8SP
TB10.4+ CD8SP

Figure 7

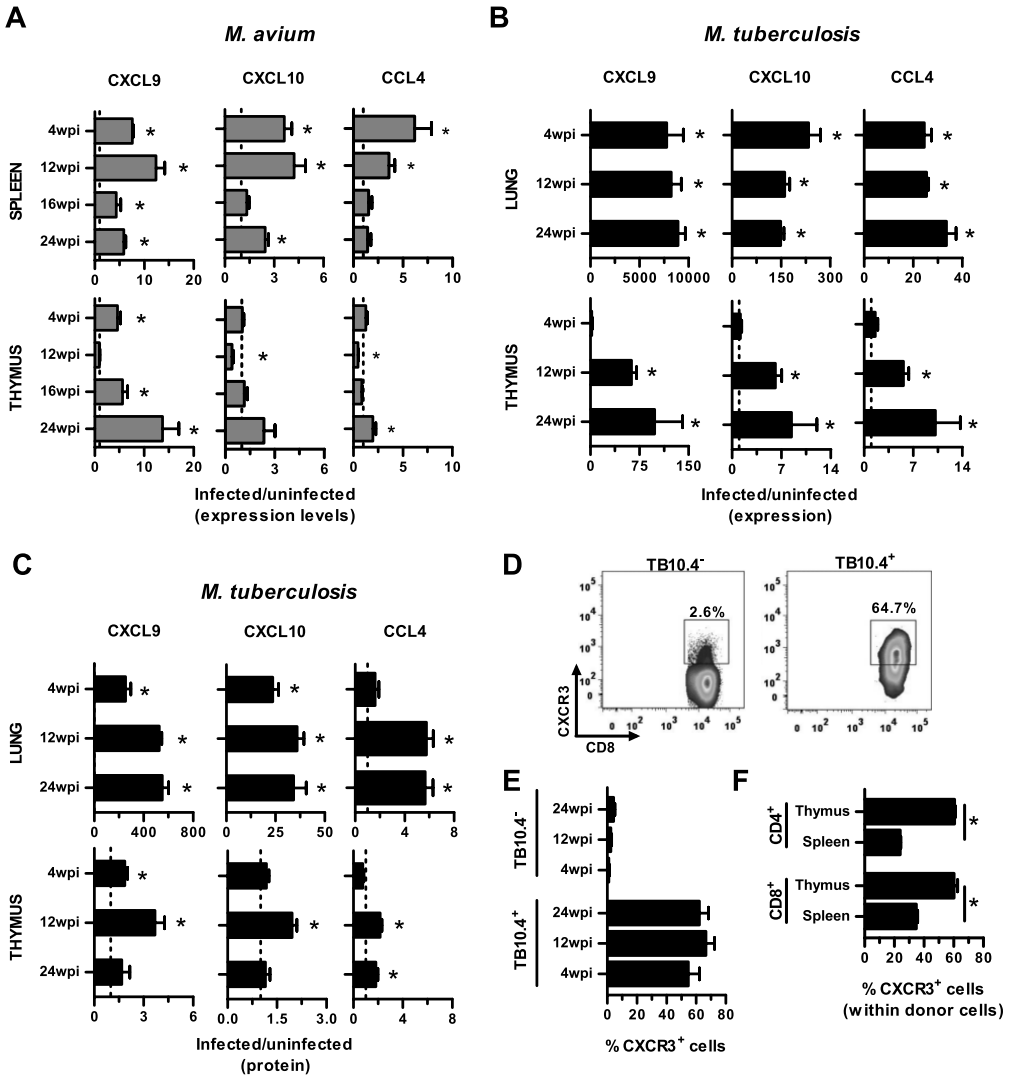
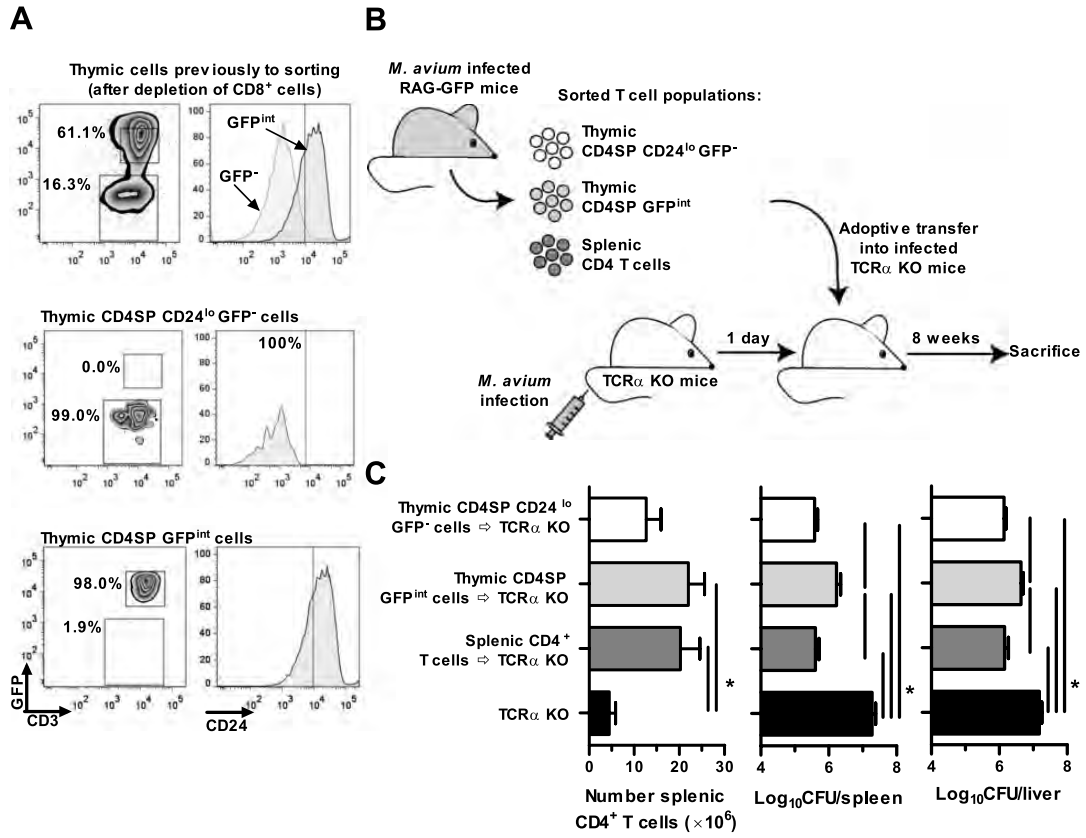
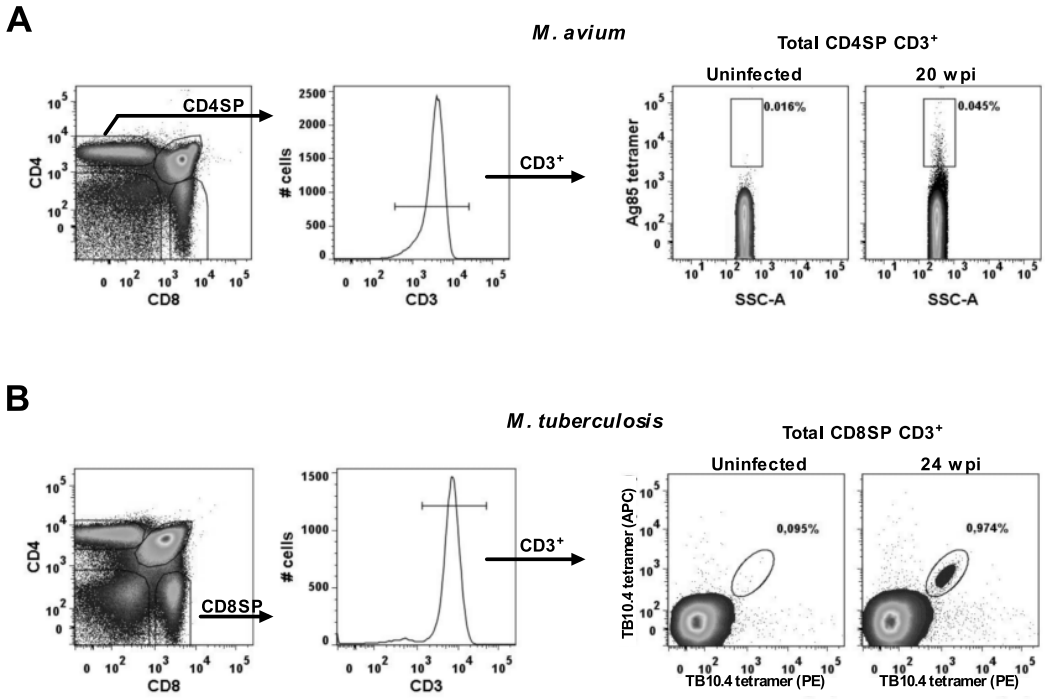

A
M. avium
CXCL9
CXCL10
CCL4
SPLEEN
THYMUS
4wpi
12wpi
16wpi
24wpi
Infected/uninfected
(expression levels)
B
M. tuberculosis
LUNG
THYMUS
Infected/uninfected
(expression)
C
M. tuberculosis
Infected/uninfected
(protein)
D
TB10.4-
TB10.4+
2.6%
64.7%
CXCR3
CD8
E
TB10.4-
TB10.4+
% CXCR3+ cells
F
CD4+
CD8+
Thymus
Spleen
% CXCR3+ cells
(within donor cells)

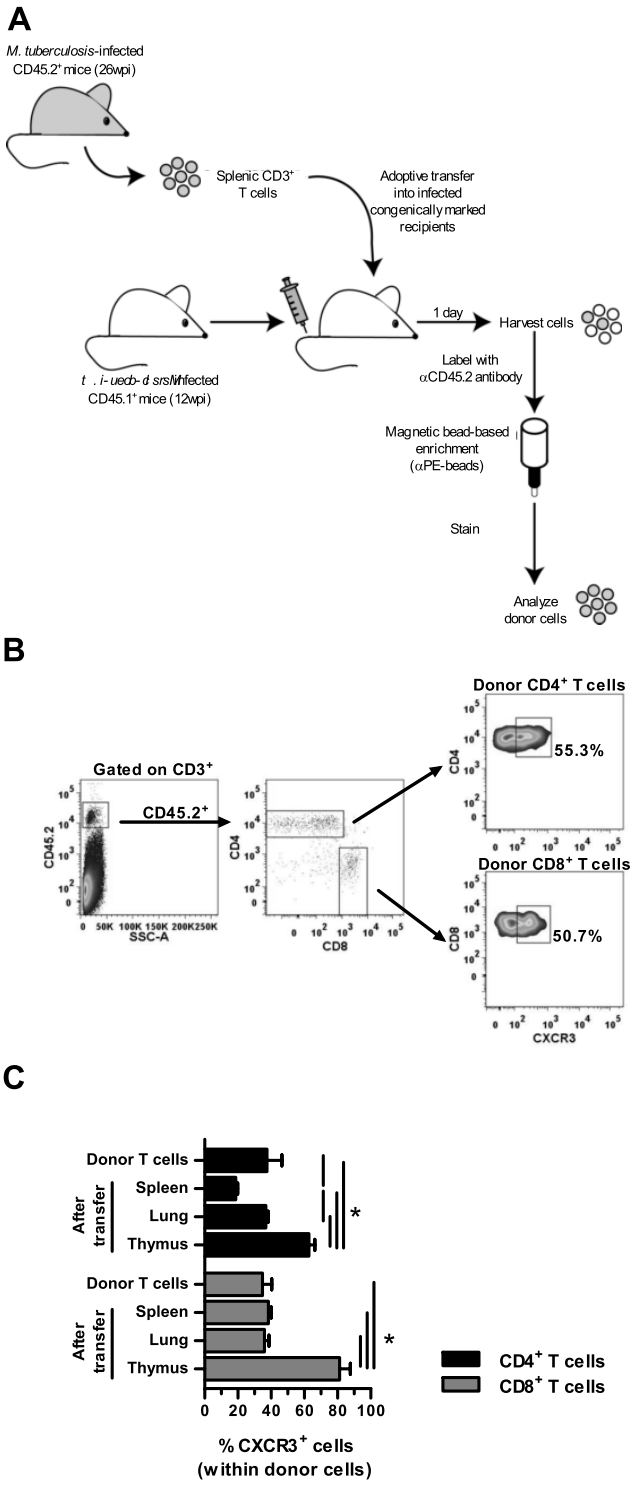
Figure 8


A
Thymic cells previously to sorting
(after depletion of CD8+ cells)
61.1%
16.3%
GFPint
GFP-
Thymic CD4SP CD24lo GFP- cells
0.0%
99.0%
100%
Thymic CD4SP GFPint cells
98.0%
1.9%
GFP
CD3
CD24
B
M. avium infected
RAG-GFP mice
Sorted T cell populations:
Thymic
CD4SP CD24lo GFP-
Thymic
CD4SP GFPint
Splenic
CD4 T cells
Adoptive transfer
into infected
TCRα KO mice
1 day
8 weeks
Sacrifice
M. avium
infection
TCRα KO mice
C
Thymic CD4SP CD24 lo
GFP- cells ⇨ TCRα KO
Thymic CD4SP
GFPint cells ⇨ TCRα KO
Splenic CD4+
T cells ⇨ TCRα KO
TCRα KO
*
0
10
20
30
Number splenic
CD4+ T cells (×10^6)
4
6
8
Log10CFU/spleen
Log10CFU/liver

Supplementary figure 1



Supplementary figure 2



The second part of this thesis focuses on the role of $CD8^+$ T cells during tuberculosis. The first chapter of this section is a review on our current knowledge about $CD8^+$ T cell function following infection. The second chapter contains only unpublished data and reports our studies with a new mouse model whose T cells are specific for TB10, an immunodominant $CD8^+$ T cell epitope of *M. tuberculosis*. Together, these studies start to clarify how $CD8^+$ T cells modulate protective immunity against *M. tuberculosis*

Part II

$CD8^+$ T cell responses to tuberculosis

retrogenic mice as a new mouse model to study $CD8^+$ T cells, their protective capacity and their mechanism of protection

Historically, two aspects of immunity to tuberculosis have been the focus of attention of multiple studies: the role of $CD4^+$ T cells in controlling infection; and the ability of the host to produce IFNɣ. While it is indisputable that both are essential components of successful immune responses to infection, other immunological features contribute to optimal immunity against tuberculosis. In this chapter, we review studies on the role of $CD8^+$ T cells in infected people and experimental animal models of tuberculosis, and summarize what is known about what constitutes protective immunity against tuberculosis and how $CD8^+$ T cells protect against *M. tuberculosis* infection.

Chapter 4

$CD8^+$ T cell responses to tuberculosis

Brief introduction to the role of $CD8^+$ T cells during tuberculosis

(unpublished)

Introduction

Tuberculosis is a major health problem and no other infectious agent is responsible for more deaths worldwide that *M. tuberculosis*[1]. The HIV/AIDS epidemic has led to the aggravation of this scenario, but has also revealed the essential role that adaptive immunity plays in the control of *M. tuberculosis*[2]. Therefore, there is great interest in clarifying which T cell subsets mediate immunity during tuberculosis, what are the protective mechanisms responsible for *M. tuberculosis* control, and whether vaccination can induce such mechanisms and be suitable to control infection.

Historically, CD4$^+$ T cells have been the focus when studying adaptive immune responses to tuberculosis, and there is no question concerning their essential role in controling infection and preventing recrudescence, in both humans and experimental animal models[3]. However, CD8$^+$ T cells are also elicited following infection, are recruited to the lung, participate in granuloma formation, and are essential for optimal immunity against *M. tuberculosis*[4]. Interestingly, how CD8$^+$ T cells contribute to protection, whether they have any unique effector function, or whether they are a suitable target for vaccination are all unanswered questions that require further investigation.

CD8$^+$ T cells are required for optimal immunity against *M. tuberculosis*

Despite all the open questions about the role of CD8$^+$ T cells during *M. tuberculosis* infection, it is now clear that they are an important component of functional immune responses to tuberculosis. The first real confirmation that these cells are required in the response to tuberculosis came from the observation, made by Flynn et al., that β_2-microglobulin (β_2m) knockout mice are extremely susceptible to intravenous (IV) infection with *M. tuberculosis*[5]. Because β_2m is essential for the assembly and trafficking of the class I heavy chain, these mice do not express MHC-I. As a consequence of the lack of MHC-I expression in the thymus, which is essential for thymic selection, these mice lack CD8$^+$ T cells. These results were confirmed by studies in mice lacking the expression of transporter associated with antigen-processing (TAP)-1$^{-/-}$, CD8$\alpha^{-/-}$ mice and K^bD^b (class I MHC heavy chain)$^{-/-}$ mice, all of which have impaired resistance to *M. tuberculosis* infection[6-11].

These studies using knockout mice substantiate a role for CD8$^+$ T cells during tuberculosis, and have been confirmed by other experimental approaches, such as

antibody-mediated depletion and adoptive cell transfers. For example, CD8$^+$ T cell depletion has been shown to impair bacterial control following IV infection with *M. tuberculosis* virulent strain H37Rv and also BCG[12]. In a different study, the susceptibility of mice after CD8$^+$ T cell depletion was very comparable to genetic ablation of MHC-I. Interestingly, CD8$^+$ T cell depletion accentuated the effects of CD4$^+$ T cell depletion following aerosol infection of mice[13]. The protective capacity of CD8$^+$ T cells has also been confirmed by adoptive transfer of either naïve or immune CD8$^+$ T cells into infected recipients. Ian Orme's group showed that CD8$^+$ T cells purified from the spleens of IV infected mice were protective when transferred to *M. tuberculosis*-infected sublethally irradiated mice[14]. Tascon et al. showed that naïve CD8$^+$ T cells protect athymic recipients after intraperitoneal (IP) infection with *M. tuberculosis*[15]. Interestingly, in this study the protective capacity of CD8$^+$ T cells was comparable to that of CD4$^+$ T cells, although mice were only analyzed at 3 weeks post-infection. In the study of Ian Orme's group, CD8$^+$ T cells significantly protected mice upon high-dose aerosol challenge, but CD4$^+$ T cells were significantly better at transferring protection during both IV and low-dose aerosol infections[14]. Studies from our own lab have confirmed that adoptive transfer of effector CD8$^+$ T cells from infected mice into sub-lethally irradiated recipients confers protection against subsequent aerosol challenge with virulent *M. tuberculosis*[16], and this model has been used to address the protective mechanisms responsible for CD8$^+$ T cell protection (described below).

Whether CD8$^+$ T cells are important in the response against *M. tuberculosis* in humans is less well defined, but several observations support the notion that CD8$^+$ T cells have a protective role following infection. For example, CD8$^+$ T cells have been described as a component of granulomas[17], and CD8$^+$ T cell clones have been generated from the pleural fluid of tuberculosis patients but also from BCG-vaccinated individuals as well as patients with latent infection[18-23]. In addition, several of these T cell clones have been shown to be able to recognize, and more importantly, kill infected macrophages[23,24]. Finally, several studies have focus on the characterization of *M. tuberculosis*-specific epitopes recognized by CD8$^+$ T cells, expanding the arsenal of research tools available to track antigen-specific T cells following infection. These reagents will make possible future studies on the function of CD8$^+$ T cells and their importance both during natural immunity to tuberculosis as well as during vaccination studies.

CD4$^+$ T cells versus CD8$^+$ T cells – historical bias?

It is undisputable that MHC-II restricted CD4$^+$ T cells are critical for host resistance against *M. tuberculosis* infection, in both humans and experimental animal models. However, one can argue against the notion that MHC-I restricted CD8$^+$ T cells are less important during infection. This perception is founded on the observation that mice lacking CD4$^+$ T cells (either genetically or following antibody-mediated depletion) are more susceptible to tuberculosis than mice lacking CD8$^+$ T cells[13]. However, it is now known that CD4$^+$ T cell help is required for optimal CD8$^+$ T cell effector function and memory generation[25]. Therefore, it is possible that CD4$^+$ T cells are important *per se*, but also as modulators of CD8$^+$ T cell activity. To support this idea, mice treated with depleting antibodies against CD4$^+$ and CD8$^+$ T cells are more susceptible than mice depleted of CD4$^+$ T cells alone, suggesting that CD8$^+$ T cells play a non-redundant role even during the initial stages of immune responses[13]. Also, the absence of CD4$^+$ T cells has been shown to impair the generation of cytotoxicity of CD8$^+$ T cells during tuberculosis[26]. Furthermore, CD4$^+$ and CD8$^+$ T cells might be necessary at different stages of infection, with CD4$^+$ T cells being important during the acute phase and CD8$^+$ T cells controlling infection during latency. This scenario is supported by experimental data on a model of antibiotics-induced latency. In this model, mice are treated with antibiotics to control disease progression, but when treatment is stopped disease reactivation occurs. Interestingly, treatment with anti-CD4 exacerbated disease, but only during the acute phase, and not during the latent phase. The reverse was true for anti-CD8 treatment, which did not influence disease severity during the acute phase, but exacerbated it during the latent stage[12].

Finally, CD4$^+$ T cells and CD8$^+$ T cells might have distinct and complementary functions during the course of infection. For example, CD8$^+$ T cells are unique in their ability to directly kill infected cells through the production of cytotoxic molecules, such as granzymes and perforin in mice, and also granulysin in humans, or by the TNF or Fas / Fas ligand (FasL) pathways[27]. CD8$^+$ T cells are theoretically capable to detect every infected cell, as almost every nucleated cell in the body expresses MHC-I, and have been shown to preferentially recognize heavily infected cells[28]. In contrast, CD4$^+$ T cell activitation is restricted to APCs expressing MHC-II, and *M. tuberculosis* has been shown to modulate MHC-II expression following infection, which could turn these cells “invisible” to host CD4$^+$ T cell surveillance[29]. Therefore it is possible that CD4$^+$ T cells and CD8$^+$ T cells have non-redundant, co-operative roles following infection, and that these change overtime as disease progresses.

M. tuberculosis antigens recognized by CD8$^+$ T cells

The idea that CD4$^+$ T cells are more important than CD8$^+$ T cells during *M. tuberculosis* infection has been reinforced by the elucidation of the mechanisms governing antigen presentation to these distinct cell types. CD4$^+$ T cells recognize mostly exogenous antigens derived from proteins that are taken up by professional APCs by phagocytosis, degraded into peptides within specialized endocytic compartments, and loaded into MHC-II. On the other hand, CD8$^+$ T cells recognize mostly antigens derived from proteins present in the cytosol, that are degraded by proteasomes, and loaded into MHC-I. Because *M. tuberculosis* resides primarily in the phagosome of infected macrophages, it is easy to envision how peptide loading into MHC-II and presentation to CD4$^+$ T cells occurs. In contrast, how *M. tuberculosis* antigens end up being presented by MHC-I to CD8$^+$ T cells has been more difficult to understand and has impact on antigen discovery.

There are at least three possible mechanisms by which *M. tuberculosis* antigens end up loaded into MHC-I and presented to CD8$^+$ T cells. These are cross-presentation; bacterial escape to the cytosol; and antigen "leakage" to the cytosol[30]. Cross-presentation refers to the ability of APCs to transfer antigens from the endocytic compartment to the cytosol, where they are processed by proteasomes and loaded into the MHC-I pathway. Although cross-presentation has been described during a multitude of scenarios, the precise molecular mechanisms by which it occurs are mostly unclear. Interestingly, our studies have revealed a role for cross-presentation following *M. tuberculosis* infection, and a connection between the cell death modality of the infected macrophage and the ability to generate CD8$^+$ T cell responses[31]. We have shown that *M. tuberculosis* modulates the synthesis of lipid mediators within the infected cell, stimulating necrosis, and inhibiting apoptosis. In addition, infection of genetically modified macrophages that are prone to die by apoptosis leads to an earlier generation of CD8+ T cell responses in a process dependent on the presence of DC, indicative of cross-presentation[31]. A second possibility is that *M. tuberculosis*, or *M. tuberculosis* proteins, end up in the cytosol, where they are subject to degradation and presentation through the MHC-I pathway. In support of this hypothesis, *M. tuberculosis* has been shown to translocate from phagosomes to the cytosol[32]. Also, *M. tuberculosis* has been shown do disrupt host cell membranes, which is likely to include phagosomal membranes[33]. Such damage could allow bacterial proteins to leak to the cytosol. Alternatively, bacterial secretion

systems could be responsible for active transport of *M. tuberculosis* proteins from the phagosome to the cytosol.

Although it is still unclear how *M. tuberculosis* antigens end up being loaded on MHC-I proteins, it is undisputable that they do, and that antigen-specific CD8$^+$ T cell responses are elicited upon infection. Although very few epitopes have been defined so far, it is clear that most antigens recognized by CD8$^+$ T cells during tuberculosis are derived from *M. tuberculosis* secreted proteins with small molecular weight[4]. This observation is consistent with the hypothesis that these proteins cross the phagosome membrane and end up in the cytosol. Therefore, members of the ESAT6 family, the Ag85 complex, the 19-kDa, 16-kDa and 38-kDa proteins and the Mtb32c protein are very common immunogens, in both people and mice (these epitopes are reviewed in extensive detail by *Woodworth et al.*[4]). Currently, there are five well-characterized *M. tuberculosis* epitopes recognized by murine CD8$^+$ T cells: $TB10_{4\text{-}11}$, $TB10_{20\text{-}28}$, $CFP10_{33\text{-}39}$, $32c_{309\text{-}318}$ and $EspA_{150\text{-}158}$ (Table 1). Among these, our studies focus mostly on $TB10.4_{4\text{-}11}$.

Table 1: Immunodominant *M. tuberculosis* antigens recognized by murine CD8$^+$ T cells

Antigen	Epitope	Sequence	Restriction	Gene	CD8 response
CFP10	33-39	VESTAGSL	K^K	Rv3674	25-30%
TB10	20-28	GYAGTLQSL	K^D	Rv3019c	30-35%
EspA	150-158	AYLVVKTLI	K^D	Rv3616	8-15%
TB10	4-11	IMYNYPAM	K^B	Rv3019c	30-50%
32c	309-318	GAPINSATAM	D^B	Rv0125	6-12%

Immunodominant CD8$^+$ T cell responses following *M. tuberculosis* infection: TB10 as a case study

CD8$^+$ T cell responses against most *M. tuberculosis* antigens are extremely immunodominant, i.e., focused on relatively few of all possible epitopes. This is best exemplified by TB10-specific responses in B6 mice, in which the majority of the CD8$^+$ T cells in the infected lung are restricted to the same H-2^b epitope, IMYNYPAM. This linear aminoacid sequence is found in two *M. tuberculosis* proteins, TB10.3 and $TB10.4^{34\text{-}37}$. TB10.4 (EsxH) is a small, secreted protein encoded within the ESX3 locus, which forms a heterodimer with its partner EsxG. TB10.3 is encoded within the ESX5 locus and is highly homologous to TB10.4. Because these proteins share the

same immunodominant epitope, from now on we will not distinguish between TB10.3 and TB10.4, and instead will use the terminology “TB10” to refer to both proteins (Table 2).

Table 2: Comparison of TB10 proteins

Protein	TB10.3	TB10.4
Gene	Rv3019c	Rv0288
Alt. name	EsxR	EsxH
Complex	EsxRS	EsxGH
Locus	ESX5	ESX3
Function	Unknown	Fe/Zn

TB10-specific CD8+ T cell frequencies after low dose aerosol infection range from 30-50% of the CD8+ T cells in the lungs of infected B6 mice, as measured by using peptide-loaded H-2 tetramers[16,31,38]. Why infected animals respond to such a complex organism (*M. tuberculosis* has approximately 4000 genes) with large expansions of CD8+ T cells to a few immunodominant epitopes is unclear. Also, it is unclear if immunodominance is beneficial or detrimental to the host. Among the current debated hypothesis are that immunodominance is determined by the host versus being driven by the bug[39]. The possibility that immunodominance is host dependent implies that T cells capable of recognizing immunodominant epitopes are over represented in the naïve repertoire, and argues for a protective role for such epitopes. Of note, this is not the case for TB10, as we have shown that the precursor frequency of these immunodominant CD8+ T cells is comparable to the one of subdominant epitopes[40]. Furthermore, modulation of the precursor frequency prior to infection by the means of vaccination could not reverse immunodominance, indicating that infection, rather than the host, determines this phenomenon[40]. One way *M. tuberculosis* could modulate immunodominance is based on the kinetics (early versus late) or amount (abundant versurs rare) of antigen production. In fact, it has been reported that *M. tuberculosis* antigen-coding sequences are over-conserved in the genome, and this has been suggested to indicate that such antigens could serve as decoy antigens, promoting long term bacterial persistence[41]. Interestingly, TB10 antigens have been excluded from such analysis, as they are among the most variable genes within the *M. tuberculosis* genome.

Although our understanding of the mechanisms governing immunodominance is still minimal, the identification of such epitopes has allowed the development of tools for the detection of CD8$^+$ T cells *in vivo* and made possible the study of the roles of these cells during tuberculosis.

Effector functions of CD8$^+$ T cells – cytokines vs cytotoxicity

How CD8$^+$ T cells protect against *M. tuberculosis* infection is still largely unknown. Answering this question has also been difficult because we still do not understand what constitutes protective immunity against tuberculosis, even for CD4$^+$ T cells. Among the existing studies, the ability to produce IFNγ and TNF are the most commonly measured features of T cells following infection. This is because these cytokines seem to act synergistically to activate infected macrophages to control bacterial replication. IFNγ and TNF act by inducing the production of nitrogen and oxygen radicals with antimicrobial capacity[2]. The essentiality of both IFNγ and CD4$^+$ T cells in the control of tuberculosis has led to the notion that IFNγ production by CD4$^+$ T cells is the single defining feature of protective immunity during *M. tuberculosis* infection. Interestingly, CD8$^+$ T cells also produce IFNγ and TNF following infection, both in people and experimental animal models[4,24,42]. Despite these observations, few studies have addressed the role of IFNγ and TNF production by CD8$^+$ T cells during the course of infection. Tascon et al., showed that adoptive transfer of naïve CD8$^+$ T cells from WT mice, but not those from IFNγ$^{-/-}$ mice, protect athymic mice from IP infection[15]. These data show that IFNγ production by CD8$^+$ T cells can mediate protection, even in the absence of CD4$^+$ T cells. However, this study fails to address if this is true in the presence of an intact CD4$^+$ T cell compartment, and whether this is the most important function of CD8$^+$ T cells in intact mice. Of notice, a different study suggested that CD8$^+$ T cells, even when capable of producing IFNγ, cannot substitute long-term for CD4$^+$ T cells[43]. As for TNF production, its importance for CD8$^+$ T cell function during *M. tuberculosis* infection has not been addressed.

Although IFNγ is critical for host defense against *M. tuberculosis*, several recent studies have emphasized the notion that IFNγ does not correlate with protective immunity, and that several IFNγ-independent mechanisms can contribute to host defense against tuberculosis. This seems particularly relevant for CD8$^+$ T cells, which have the potential to be cytotoxic. In fact, cytotoxic CD8$^+$ T cells have

been described in both people and experimental animal models, and the mechanisms responsible for CTL activity have been experimentally addressed[16,23,24,44]. Interestingly, CD8$^+$ T cells seem to rely on multiple mechanisms to kill *M. tuberculosis* infected targets, such as Fas and TNF mediated killing and granule exocytosis involving granzyme and perforin[16]. In addition, granulysin – a component of CTL granules in people, but not present in mice – has been shown to directly kill extracellular *M. tuberculosis* as well as intracellular *M. tuberculosis* in a perforin-dependent manner[45]. These data are supported by the observation that perforin$^{-/-}$ mice show decrease survival following aerosol *M. tuberculosis* infection[8,10].

The observations that CD8$^+$ T cells can produce IFNγ, TNF, and express multiple mechanisms of cytotoxicity establish the idea that CD8$^+$ T cells are extremely heterogeneous during *M. tuberculosis* infection. This notion is supported by the detection of distinct populations of IFNγ-producing and cytolytic CD8$^+$ T cells during infection[46], and our unpublished observations that only 10-15% of TB10-specific CD8$^+$ T cells express IFNγ. Of notice, heterogeneity in gene expression at the single-cell level is observed during other antigen-specific CD8$^+$ T cell responses. This is the case during infection with influenza virus and *Listeria monocytogenes*, two settings in which CD8$^+$ T cells are crucial, where only a minority of responding CD8$^+$ T cells coordinately expressed both perforin and granzymes[47]. These data suggest that the lack of coordinated expression of “killer genes” can limit the number of competent CTL and impair host protection, a possibility that should be addressed during *M. tuberculosis* infection. In addition, CD8$^+$ T cells are slow to upregulate perforin expression upon stimulation[48], suggesting that competent CTL might emerge later during infection, rather than during primary responses. Collectively, these data suggest that the polarization of CD8$^+$ T cell subsets following *M. tuberculosis* challenge could result from the emergence of two distinct populations – cytokine-producing cells during initial responses, and CTLs during latent stages – and their relative protecting capacities should be experimentally addressed.

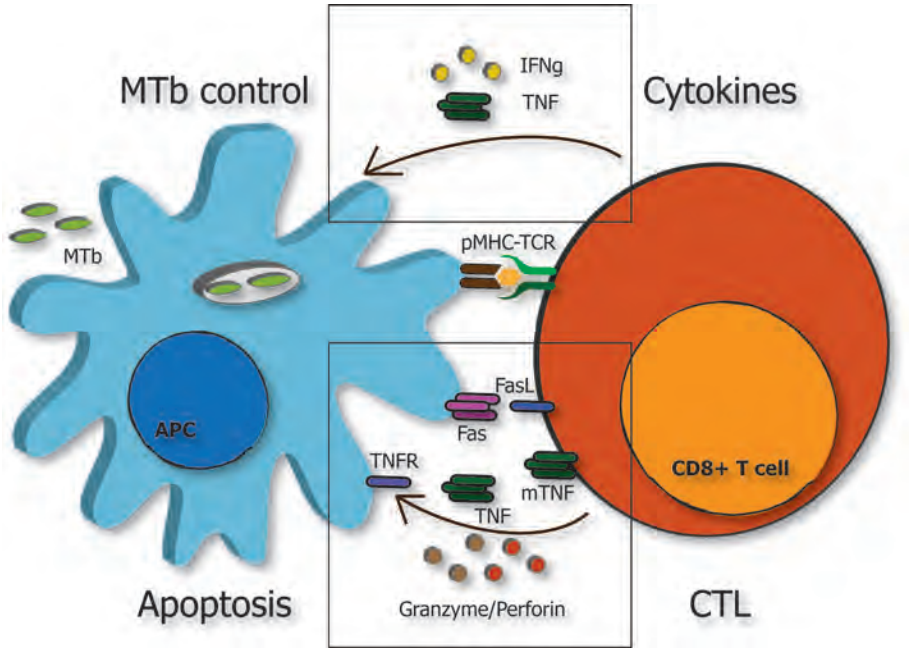


Figure 1: Mechanisms of protection by CD8+ T cells. CD8+ T cells can be either cytotoxic or produce pro-inflammatory cytokines. Production of IFNγ and TNF stimulates the infected macrophage to control bacterial growth, while CTL activity induces apoptotic cell death of the infected cell. CTL activity can occur by different mechanisms, such as through cytotoxic granules such as granzyme and perforin; Fas/FasL mediated killing; or TNF mediated cell death.

Open questions

Although several studies have demonstrated that CD8+ T cells are essential for optimal immunity to tuberculosis, different aspects of CD8+ T cell biology remain a mystery. One of the reasons for this is the lack of an animal model to serve as a source of clonotypic CD8+ T cells specific for *M. tuberculosis* antigens. Such a model would allow studies on the priming and differentiation of CD8+ T cells following *M. tuberculosis* infection, their acquisition of effector functions, and on the molecular mechanisms responsible for CD8+ T cell-mediated protection. These studies are essential to understand how CD8+ T cells protect against tuberculosis; at which stage of infection are they most important; whether they perform any unique effector function; what is the contribution of cytokine production versus cytotoxicity for CD8+ T cell mediated protection; and whether vaccine-elicited CD8+ T cells can confer protection against tuberculosis, an essential condition for rational vaccine design. We

believe that such a model would be an invaluable tool to understand what constitutes protective immunity to tuberculosis, and creating such mouse was the overarching goal of this section of my thesis.

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The generation of a successful adaptive immune response is essential for control of infection with *M. tuberculosis*. However, despite the central role played by T cells during tuberculosis, how they control infection and what constitutes protective immunity against *M. tuberculosis* is still unclear. Among these, $CD8^+$ T cells are essential for optimal immunity during infection, but the mechanisms by which they control bacterial replication are largely unknown. Here, we generate a new mouse model, carrying $CD8^+$ T cells specific for the immunodominant antigen TB10, and address how these cells mediate protective immunity during tuberculosis.

Chapter 5

Retrogenic mice expressing $CD8^+$ T cells specific to the immunodominant *M. tuberculosis* antigen TB10

A new mouse model to study $CD8^+$ T cell responses following *M. tuberculosis* infection

(unpublished)

Abstract

Immunity to *Mycobacterium tuberculosis* infection relies on the establishment of a successful adaptive immune response, with T cells playing a central role in controlling infection. Among these, $CD8^+$ T cell responses are detected in both infected people and experimental animal models of tuberculosis, and $CD8^+$ T cells are required for optimal immunity against virulent *M. tuberculosis*. To elucidate the role of $CD8^+$ T cells during tuberculosis, we developed a novel mouse model with a high frequency of antigen-specific $CD8^+$ T cells that recognize the immunodominant antigen TB10 (EsxH; Rv0288). By combining single cell sorting of tetramer positive cells and PCR amplification of T cell receptors (TCR), we identified and cloned four distinct TCRs specific for $TB10.4_{4\text{-}11}$. These four individual TCRs were expressed in mice, using retrovirus-mediated stem cell gene transfer. The resulting retrogenic (Rg) mice ("retro" from retrovirus, "genic" from transgenic) have a high frequency of T cells that express the recombinant TCR. We show that these T cells recognize and respond to TB10, and are able to mount protective immune responses against *M. tuberculosis*. To investigate the mechanism(s) leading to bacterial control by the protective clones, we generated Rg mice whose cells are unable to secrete IFNγ ($IFN\gamma^{-/-}$ Rgs) or incapable of perforin-mediated killing ($Prf^{-/-}$ Rgs). After adoptive transfer into irradiated recipients, we find that $CD8^+$ Rg T cell mediated protection requires IFNγ, but does not require perforin. We believe this new Rg mouse model, the first expressing $CD8^+$ T cells specific for an *M. tuberculosis* epitope, will be useful for defining the role of $CD8^+$ T cells during infection and the mechanism(s) underlying their protective capacity.

Introduction

Despite the availability of multiple anti-tuberculosis drugs and the existence of a vaccine – BCG – the human pathogen *Mycobacterium tuberculosis* is still a major health problem, killing approximately 1.5 million people every year and infecting one third of the world's population[1]. The continuing HIV/AIDS epidemic and the spread of multi-drug resistant strains has led to the perpetuation of the worldwide tuberculosis epidemic. Interestingly, the majority of infected individuals do not develop active disease, and instead remain in an asymptomatic state termed latent infection. *M. tuberculosis* control involves the establishment of a protective adaptive immune response, with T cells playing a key role in this process[2]. Consequently, there is great interest in determining which T cell subsets mediate anti-mycobacterial immunity, delineating their effector functions, and evaluating whether vaccination can elicit these T cell subsets and induce protective immunity.

While CD4$^+$ T cells are essential for immunity to tuberculosis[2], CD8$^+$ T cell responses are detected in both infected people and experimental animal models of tuberculosis, and CD8$^+$ T cells are recruited to the infected lung and required for optimal immunity against virulent *M. tuberculosis*[3]. However, what constitutes protective immunity against tuberculosis is still largely undefined, and how CD8$^+$ T cells protect against *M. tuberculosis* infection is still unknown.

The most commonly documented feature of T cells following infection is their ability to produce pro-inflammatory cytokines – such as IFNγ and TNF. IFNγ and TNF induce the production of oxygen and nitrogen radicals by infected macrophages, and these compounds have antimicrobial activity, leading to bacterial control[4,5]. Indeed, mice that are unable to produce IFNγ are extremely susceptible to *M. tuberculosis* infection[6], and people with genetic defects in the IFNγ signaling pathway are incredibly susceptible to mycobacterial infections[7]. However, despite being critical for host defense, several recent studies have demonstrated that IFNγ levels do not correlate with protective immunity against *M. tuberculosis*[8,9], and several IFNγ-independent mechanisms that contribute to host defense against tuberculosis have been described[10,11]. This observation seems particularly relevant for CD8$^+$ T cells, which in addition to their ability to produce cytokines have the potential to mediate direct cell killing. In fact, cytotoxic CD8$^+$ T cells have been described in both people and experimental animal models[12-14] and mice lacking components of cytotoxic granules, such as perforin, are more susceptible to aerosol *M. tuberculosis* infection[15,16].

Although several studies have demonstrated that CD8$^+$ T cells are essential for optimal immunity to tuberculosis, how CD8$^+$ T cells mediate protection, at which stage of infection are they most important and what is the contribution of cytokine production versus cytotoxicity for CD8$^+$ T cell mediated protection is still unclear.

To answer such questions, we generated a set of retrogenic (Rg) mice that express a high frequency of CD8$^+$ T cells specific for the immunodominant *M. tuberculosis* antigen TB10. We show that CD8$^+$ T cells are primed in the draining lymph node around d11 after infection, acquire an activated phenotype very shortly after priming, and are capable of protecting mice against *M. tuberculosis* infection. Furthermore, we address the contribution of cytokine production versus cytotoxicity for the protection conferred by CD8$^+$ T cells and demonstrate that IFNγ, but not perforin, is essential for CD8$^+$ T cell mediated protection following infection.

Materials and methods

Mice

C57BL/6 (WT), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I), B6.SJL-Ptprc^{a}Pepc^b/BoyJ (CD45.1), B6.129S7-Ifng^{tm1Ts}/J (IFN$\gamma^{-/-}$), C57BL/6-Prf1^{tm1Sdz}/J (Prf$^{-/-}$) and B6.129S2-Tcra^{tm1Mom}/J (TCR$\alpha^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Limited and Vα2var mice[17] were bred in our facilities. Mice were 7 to 10 weeks old at the start of the experiments. All animal experiments were performed in accordance with National and European Commission guidelines for the care and handling of laboratory animals and were approved by the National Veterinary Directorate and by the local Animal Ethical Committee or by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines. Mice infected with *M. tuberculosis* were housed in a biosafety level 3 facility under specific pathogen-free conditions at the Animal Biohazard Containment Suite (Dana Farber Cancer Institute, Boston, MA).

Experimental infection

For each *M. tuberculosis* (Erdman strain) infection, a bacterial aliquot was thawed, sonicated twice for 10 s in a cup horn sonicator, and then diluted in 0.9% NaCl–0.02% Tween 80. A 15 ml suspension of *M. tuberculosis* was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technologies) and mice were infected via

the aerosol route using a nose-only exposure unit (Intox Products) and received 100–200 CFU/mouse. At different times post-infection, mice were euthanized by carbon dioxide inhalation or by decapitation and organs were aseptically removed, individually homogenized and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H11 agar plates. Plates were incubated at 37ºC and bacterial colonies were counted after 21 d. For survival experiments, animals were euthanatized when displaying any of the following signs: marked loss of appetite and fluid intake or staring coat, hunched posture and subdue behaviour or severe weight loss over a period of more than 3 days. When indicated, "Log protection" refers to the difference between the log of the bacterial load in the lung of control mice and the log of the bacterial load in the lung of the remaining experimental groups.

Flow cytometry

Surface staining was performed with antibodies specific for mouse CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), Vα2 (B20.1), Vβ5 (MR9-4), Vβ11 (KT-11) (from Biolegend, CA, USA, BD Pharmingen, CA, USA, or eBiosciences, CA, USA). The tetramers TB10.$4_{4–11}$-loaded H-2 K^b (PE and APC labeled) were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA, USA); staining with the tetramer was performed for 20 min on ice during the incubation with antibodies for the surface staining. All cells were fixed before acquisition with 2% formaldehyde in PBS for 30 min. Cell analysis was performed on a FACS Canto or Canto II using FACS Diva Software (Becton Dickinson, NJ, USA). Data were analyzed using FlowJo Software (Tree Star, OR, USA). Single-lymphocyte events were gated by forward scatter versus height and side scatter for size and granularity.

Cell preparation and *in vitro* stimulation and IFNγ measurement by intracellular cytokine staining (ICS)

Cell suspensions from spleen and lymph nodes were prepared by gentle disruption of the organs through a 70 µm nylon strainer (Fisher). For lung preparations, tissue was digested for 1 h at 37 °C in 1 mg/mL collagenase (Sigma) prior to straining. Erythrocytes were lysed using a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM sodium EDTA pH 7.2) and, after washing, cells were resuspended in supplemented DMEM or RPMI (10% heat inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml streptomycin and 50 U/ml penicillin,

all from Invitrogen). Cells were enumerated in 4% trypan blue on a hemocytometer. 0.5-1x10^6 cells were analyzed by flow cytometry, as described above. For ICS stimulations, 10^6 cells were plated in each well of a 96-well plate and incubated in the presence of TB10.4$_{4-11}$ peptide (10 μM; New England Peptide). Incubation in the presence of αCD3/αCD28 (1 ug/mL; BioLegend) or in the absence of stimuli were used as positive and negative controls, respectively. Golgi Stop solution (BD Biosciences) was added to the cultures 1 h after the start of stimulation. Cells were collected after 5 h of culture and the percentage of cells producing IFNγ was determined by intracellular cytokine staining with antibodies specific for mouse IFNγ (XMG1.2) (Biolegend) according to the manufacturer's instructions.

Single-cell sorting and PCR amplification of TB10-specific TCRs

Cell suspensions from the lungs of individual Vα2var mice infected with *M. tuberculosis* were prepared as described above. Bulk CD8$^+$ T cells were purified from each suspension using the CD8$^+$ T cell isolation kit (from Miltenyi Biotec, Germany). Magnetic separation was performed using an Auto-MACS separator (Miltenyi Biotec, Germany). CD8$^+$ T cells were stained with antibodies specific for mouse CD3, CD8, and TB10.4 tetramer, as described above. Single CD8$^+$ T cells were sorted from this suspension by standard fluorescence activated cell sorter (FACS) methods into 10 ul of 1x modified MMLV-RT buffer (2% Triton X-100 (Sigma), 0.1 mg/ml BSA (NEB), 1.5 mM each dNTPs (Ambion), 5 mM DTT (Invitrogen), 10 U RNAse Out (Invitrogen), 30 U MMLV-RT (Invitrogen), 50 nM each primer (see external primers on Table 1), under BSL3 conditions, on a FACS Aria (Becton Dickinson). Wells were covered with 30 uL mineral oil (Sigma) and plates sealed. cDNA synthesis was performed immediately after sorting, for 2 h at 37º C, followed by inactivation for 20 m at 80ºC. 1.7 uL of the resulting cDNA was used for the first nested PCR reaction. Reactions were performed separately for the T cell receptor α (TCRα) and TCRβ chains in 10 uL of 1x buffer containing 1.5 mM MgCl2, 200 nM dNTPs (Invitrogen), 200 nM each primer (see external primers on Table 1) and 2.5 units Platinum Taq polymerase (Invitrogen). Fragments were amplified for 35 cycles of 20 s denaturation at 94º C, 45 s annealing at 52º C and 1 min extension at 72º C. 2 uL of the resulting reaction was used in the second step of PCR amplification, in a 20uL reaction with identical conditions, except for the usage of the internal primer sets (Table 1) and only for 25 cycles of amplification. The resulting products were treated in 1x Shrimp Alkalyne Phosphatase buffer with 1 U SAP (USB) and 1 U exonuclease I (NEB) for 1 h at 37º C, the reaction inactivated at 80º C for 10 m, and products sequenced by using the

corresponding TCRα constant (TRAC) or TCRβ constant (TRBC) internal primers (Table 1).

Generation of retrogenic mice

Retrogenic mice were generated as previously described[18]. The TCRα and TCRβ chains were cloned with a 2A peptide-linker that allows stoichiometric expression of both chains of the TCR by using genomic DNA extracted from purified T cells from a pool of naïve WT splenocytes and the primers described in Tables 2 and 3. The 2A peptide-linked constructs were then subcloned into the pMIG vector, as previously described[18]. Retroviral producer cell lines were generated and used for retroviral transduction of murine bone marrow cells as described previously[18]. Transduced bone marrow cells were injected via the tail vein into irradiated (1200 rads) recipient WT mice, and mice were analyzed 5–6 weeks after adoptive cell transfer.

Adoptive transfer of retrogenic cells

Single cell suspensions of pools of spleens and lymph nodes from retrogenic were prepared. Cells were stimulated for 60-72 h in the presence of IL-2 (10 U/mL), IL-12 (10 U/mL) and TB10 peptide (10 uM). Fresh IL-2 and IL-12 were added after 24 h of stimulation, and fresh IL-2 was added after 48 h of stimulation. Following stimulation, CD8$^+$ T cells were purified from each suspension using the CD8$^+$ T cell isolation kit (from Miltenyi Biotec, Germany). Magnetic separation was performed with LS columns (Miltenyi Biotec, Germany). Alternatively, CD8$^+$ T cells were stained with antibodies specific for mouse CD3, CD8 and Vα2, as described above, and sorted by standard fluorescence activated cell sorter (FACS) methods. After purification cells were counted and 10^6 cells were transferred intravenously to each recipient. Recipient mice (CD45.1) were sub-lethally irradiated 4-6 hours prior to injection (600 Rads). When used as recipients, TCR$\alpha^{-/-}$ mice were not irradiated. All mice were infected with *M. tuberculosis* via the aerosol route within 24 h of cell transfer.

Statistical analysis

All data are represented as mean + SEM. Data were verified for Gaussian distribution or Mann-Whitney *U* test were performed to compare two groups. To compare more than 2 groups, one-way ANOVA, followed by Bonferroni post-hoc test was performed. Differences with a $p<0.05$ were considered significant and represented by *. Survival analysis was determined by Kaplan–Meier test, followed by a logrank (Mantel–Cox) test.

Results

Generation of a TB10-specific Rg mouse model

TB10.4 (EsxH) is a small, secreted protein encoded within the ESX3 locus, which forms a heterodimer with its partner EsxG. Highly homologous to TB10.4, TB10.3 is encoded within the ESX5 locus, and both proteins contain the H2-Kb-restricted epitope IMYNYPAM, here called TB10[19-22]. TB10-specific responses can be tracked by using loaded H2-Kb tetramers, and after low dose aerosol infection, 30-50% of the CD8$^+$ T cells in the lungs of infected B6 mice recognize TB10, defining TB10 as an immunodominant epitope[14,23-26]. To study CD8$^+$ T cell responses to *M. tuberculosis* we decided to generate Rg mice specific for TB10. We wished to obtain TB10-specific CD8$^+$ T cells that expressed the Vα2 TCR so that Rg cells could be followed in vivo by the use of TCR-specific antibodies. Therefore, we infected Limited and Vα2var mice[17] and analyzed their ability to generate immune responses against *M. tuberculosis*. These mice carry a Vα2 minilocus, ensuring that the specific TCRs recognizing TB10 would be of the Vα2 family and therefore possible to stain with available commercial antibodies. In addition to the Vα2 minilocus, Limited mice express the OT-I TCRβ chain, while Vα2var mice can pair their transgenic TCRα chain with any endogenous TCRβ chains[17].

Interestingly, we found that Limited mice were as susceptible to *M. tuberculosis* infection as mice without any T cells (TCR$\alpha^{-/-}$), while Vα2var mice were not significantly more susceptible than WT mice, despite having a reduced repertoire of T cells (Figure 1A). More importantly, CD8$^+$ T cells from Vα2var mice were capable of recognizing TB10, based on staining with specific tetramers and responded to stimulation with the cognate peptide by producing IFNγ (Figure 1B and 1C).

Since Vα2var mice are capable of recognizing TB10, we used CD8$^+$ T cells from these mice to identify TCRs specific for TB10. To do so, we sorted single cells from three infected mice and amplified and sequenced their TCRs. Strikingly, the TB10-specific repertoire was dominated by a small number of TCR sequences per individual mouse analyzed – 1 sequence for mouse 1 and 3, and 2 sequences for mouse 2 (Figure 1D). Interestingly, the complementarity determining region 3 (CDR3) of the TCRα chains of the 4 dominant clones was diverse, both in terms of length and amino acid composition, but the CDR3 of the TCRβ chains was very conserved (Figure 1E).

These 4 candidate TCR sequences were amplified, linked with 2A peptides, used to generate retrovirus-producing cells, and transfected into bone marrow stem cells, leading to the generation of 4 independent retrogenic mice. The resulting mice express a high frequency of CD8$^+$ T cells that express GFP (present in the vector) and the desired TCR (Figure 2A), and are recognized by TB10-specific tetramers (Figure 2B). Interestingly, the different Rg mice recognize TB10 with different affinities/avidities, as measured by tetramer titration on activated cells (Figure 2C). Despite the differences in tetramer binding, all retrogenic cells are capable of detecting TB10, as indicated by IFNγ production after in vitro stimulation with the TB10 peptide (Figure 2D). These data show that Rg cells are specific for, and capable of recognizing the TB10 epitope.

TB10-specific CD8$^+$ T cells are primed between d12 and d14 after infection

To study whether Rg cells are activated in vivo, we transferred naïve purified CD8$^+$ T cells from Rg TCR3 mice into congenically marked infected recipients, and analyzed the presence and activation status of the transferred cells in the draining pulmonary lymph node (pLN) and the lung at different times after aerosol infection. Our data shows that TB10-specific cells become activated between d12 and d14 after infection, as measured by the upregulation of CD44 and the downregulation of CD62L in the retrogenic cells (Figure 3A). These cells acquire the ability to produce IFNγ very rapidly following activation in the pLN, with around 30% of the Rg cells being IFNγ$^+$ at day 13 after infection (Figure 3B). Interestingly, Rg cells appear in the lung around d13 post infection, but only a small fraction expresses IFNγ upon stimulation at that time (Figures 3A and B). Collectively, these data show that Rg cells are primed in vivo following infection, and are capable of migrating to the infected lung.

TB10-specific CD8$^+$ T cells protect mice from aerosol infection with *M. tuberculosis*

After establishing that TB10-specific cells are primed in vivo and traffic to the lung, we sought to determine if these CD8$^+$ T cells are capable of protecting mice from aerosol challenge with *M. tuberculosis*. To address this question, Rg cells were differentiated in vitro with IL-2, IL-12 and TB10 peptide into an activated phenotype

(equivalent to a CD4$^+$ T cell T_h1 phenotype, and therefore referred to as a T_c1 phenotype). 10^6 T_c1 cells were transferred into sublethally irradiated mice, and cell numbers and bacterial loads determined at day 21, a time point before the recruitment of an endogenous T cell response[14]. Of note, all mice had large numbers of Rg cells within the infected lung (Figure 4A), but some Rg cells were able to confer protection, while others did not provide significant protection when compared to activated CD8$^+$ T cells recognizing an irrelevant peptide – OT-I cells recognize the ovalbumine peptide SIINFEKL, not present in *M. tuberculosis* (Figure 4B). Because even transfer of large numbers of activated OT-I cells protected mice from *M. tuberculosis* infection, we next determined how cell number correlated with protection in our adaptive transfer model, by using one of the most protective Rg TCRs, TCR3. Interestingly, transfer of as low as 10^4 Rg cells was sufficient to reduce bacterial loads in the lung of infected animals, while this effect was lost when using non-specific OT-I cells (Figure 4C). These data show that TB10-specific CD8$^+$ T cells are activated in vivo following infection, and are capable of reducing bacterial loads in the lung of infected mice. In addition, these results suggest that intrinsic TCR properties modulate the protective capacity of CD8$^+$ T cells, as TB10-specific CD8$^+$ T cells recognizing the same epitope display different degrees of protection in our model.

Protection mediated by TB10-specific CD8$^+$ T cells requires IFNγ production, but not perforin

Having determined that TB10-specific Rg cells are capable of reducing bacterial load in the lung of *M. tuberculosis* infected mice, we next addressed the mechanism responsible for the protection observed. Because CD8$^+$ T cells have been shown to both produce cytokines and be cytotoxic following *M. tuberculosis* infection, we generated mice with genetic defects in either pathway to assess the relative contribution of each of those to the protection conferred by adoptive transfer of Rg cells. Therefore, we generated TCR3 Rg mice using BM from IFNγ$^{-/-}$ or Prf$^{-/-}$ mice, and used purified CD8$^+$ T cells from those mice in our adoptive transfer model. Upon transfer of 10^6 activated cells, Prf$^{-/-}$ cells led to a reduction in CFU similar to that of WT Rg cells, while the protective capacity of IFNγ$^{-/-}$ cells was significantly reduced (Figure 5A).

Since perforin has been shown to be essential for the protective capacity of CD8$^+$ T cells in an adoptive cell transfer model similar to ours[14], we addressed whether differentiating Rg cells into T_c1 effectors could explain why CD8$^+$ T cell mediated protection was perforin-independent. To do so, naïve Rg cells were

transferred to infected TCR$\alpha^{-/-}$ mice, and animal survival was followed. In accordance with our previous results, mice receiving WT or Prf-deficient Rg cells survived longer than animals that received no cells or IFN$\gamma^{-/-}$ Rg cells (Figure 5B). These results confirm that CD8$^+$ T cells specific for a single *M. tuberculosis* antigen – TB10 – are capable of reducing bacterial loads in the lungs of infected mice, and that this protective capacity is determined, at least to a large extent, by their ability to produce IFNγ.

Discussion

Although CD8$^+$ T cells have been considered an essential component of optimal immunity during tuberculosis, how they contribution to host protection against *M. tuberculosis* is still largely undefined. One of the reasons for these shortcomings was the lack of an animal model that, similarly to the existing CD4$^+$ transgenics specific for ESAT-6[27,28] and Ag85[29], could be used to study CD8$^+$ T cell responses in infected mice. Here we present such a model, not by the means of transgenic mice, but by the generation of Rg mice carrying TCRs specific for the immunodominant *M. tuberculosis* antigen TB10. These mice express the desired TCRs, and recognize and respond to their cognate antigen. Furthermore, our data shows that TB10-specific Rg cells are primed in vivo following infection and migrate to the lung to fight *M. tuberculosis* infection. Interestingly, CD8$^+$ T cell priming seems to be slightly delayed when compared to the data generated with ESAT-6-specific CD4$^+$ transgenic T cells, reported to be primed between days 10 to 12 after infection[27,28]. If this is true, it might be one of the reasons why CD4$^+$ T cells seem to play a more important role in the early stages of infection, as CD8$^+$ T cells would only get recruited to the lung after the arrival of CD4$^+$ T cells. Another possibility is that the TB10 antigen is expressed later than ESAT-6, and this is a difference in antigen presentation rather than a difference between CD4$^+$ and CD8$^+$ T cells. Although the exact kinetics of expression of *M. tuberculosis* antigens during infection is still unclear, this hypothesis is in agreement with the observation that Ag85-specific transgenic CD4$^+$ T cells are also primed around d12 after infection[29].

Perhaps the most important finding of our studies is that CD8$^+$ T cells directed against a single epitope of *M. tuberculosis* are capable of significantly reducing the bacterial load in the lung of infected animals, even when as few as 10^4 cells are transferred into irradiated mice. To our knowledge, this is the first demonstration that

TB10-specific CD8$^+$ T cells can be protective. This data is of significant relevance since TB10 containing vaccines are being developed for use in people[30-32].

Interestingly, our data also raises concerns about TB10 vaccination, due to our observations that CD8$^+$ T cell responses specific for this antigen are (oligo)clonal, and that our different Rg clones display different efficiencies to protect mice from *M. tuberculosis* challenge. That infected animals should respond to such a complex organism (*M. tuberculosis* has approximately 4000 ORF) with large expansions of CD8$^+$ T cells to a few immunodominant epitopes is already unexpected, but that within each individual mouse each TB10-specific response is dominated by one or two TCR clones is even more striking. One possibility is that T cell priming in the pLN is so efficient that only a small number of T cell clones ever gets to contact MHC-I molecules loaded with TB10, and those clones expand to dominate the CD8$^+$ T cell response. Alternatively, active mechanisms might exist that prevent other TB10-reactive cells from proliferating. Future competition experiments with the different Rg TCRs described in this study should yield interesting answers to these questions. However, since different TCRs to the same antigen seem to differently mediate the ability of CD8$^+$ T cells to protect mice against *M. tuberculosis* challenge, vaccination strategies need to be designed with caution, in order to elicit maybe not the strongest T cell responses, but instead the most protective ones.

Finally, our data also addressed what determines a protective immune response against *M. tuberculosis* infection, at least in regard to CD8$^+$ T cells. We show that CD8$^+$ T cells require IFNγ to mediate protection, while perforin seems to be dispensable. These data are in agreement with the essential role of IFNγ during tuberculosis, and with previous publications reporting IFNγ production by CD8$^+$ T cells as a protective mechanism[33]. However, it is important to point out that studies similar to ours but using in vitro activated CD4$^+$ T cells demonstrated that CD4$^+$ T cell mediated protection was independent of IFNγ production[11]. These data suggest that CD8$^+$ T cells might be a non-redundant source of IFNγ, although their contribution in our model might be exacerbated since CD4$^+$ T cell activity is reduced or absent. Therefore, it will be important to study CD8$^+$ T cell responses in the presence of protective CD4$^+$ T cells.

Although our data is in agreement with previous publications on the essential role of IFNγ in T cell mediated protection against *M. tuberculosis*, our data is in apparent disagreement with another publication in which perforin is required for CD8$^+$ T cells to be protective[14]. One important difference between the two studies is the

source of the $CD8^+$ T cells. In this study, we use Rg cells specific for a single *M. tuberculosis* epitope, and differentiate them in vitro into T_c1 effectors. Woodworth et al. used polyclonal $CD8^+$ T cells purified from the spleen of infected animals. These cells differentiated in vivo for a long period of time, in the presence of $CD4^+$ T cells. Since $CD4^+$ T cells have been shown to be important for the development of CTL activity by $CD8^+$ T cells[34], this might account for the observed differences. Another possibility is that CTL capacity only emerges at later stages of infection, which is not mimicked by our short-term culture conditions. Therefore it will be important to understand if our Rg cells have the capacity to differentiate into CTLs, when does such differentiation occur, and whether it is dependent on the presence of $CD4^+$ T cells. Such experiments should provide valuable insight on the generation of CTLs during tuberculosis and their contribution to $CD8^+$ T cell mediated protection.

Collectively, these findings shed light on the mechanisms of protection against *M. tuberculosis* infection mediated by $CD8^+$ T cells. Understanding such mechanisms, and clarifying what constitutes protective immunity, is an essential component in the rational design of vaccines against tuberculosis.

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Figures and Tables

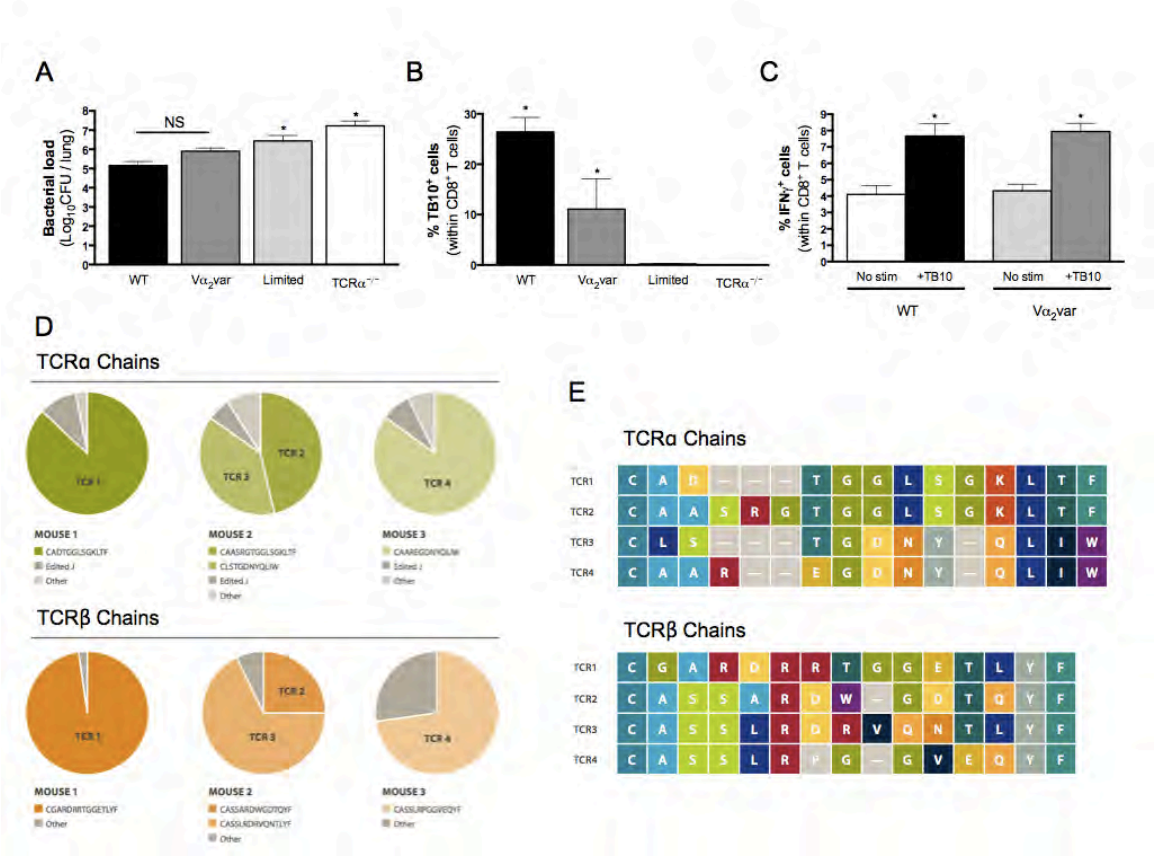

Figure 1: Selection of TB10-specific TCRs for the generation of retrogenic mice. T cells from Vα2var mice mount immune responses to *M. tuberculosis,* as assessed by bacterial load in the lungs of WT, Vα2var, Limited and TCRα$^{-/-}$ mice, 4 weeks after aerosol infection. CD8$^+$ T cells from Vα2var mice recognize TB10, determined by the presence of TB10-specific tetramer$^+$ CD8$^+$ T cells (B) and their ability to produce IFNγ following stimulation (C). TB10-specific cells are (oligo)clonal, as assessed by analysis of single cell TCR sequences of TB10-specific CD8$^+$ T cells from the lung on 3 individual Vα2var mice infected with *M. tuberculosis* (D) and analysis of the respective CDR3 aminoacid sequences of the dominant TCRs (E). * $p < 0.05$. Comparison in (A) and (B) is against the TCRα$^{-/-}$ group, and in (C) between the non-stimulated and the stimulated conditions.

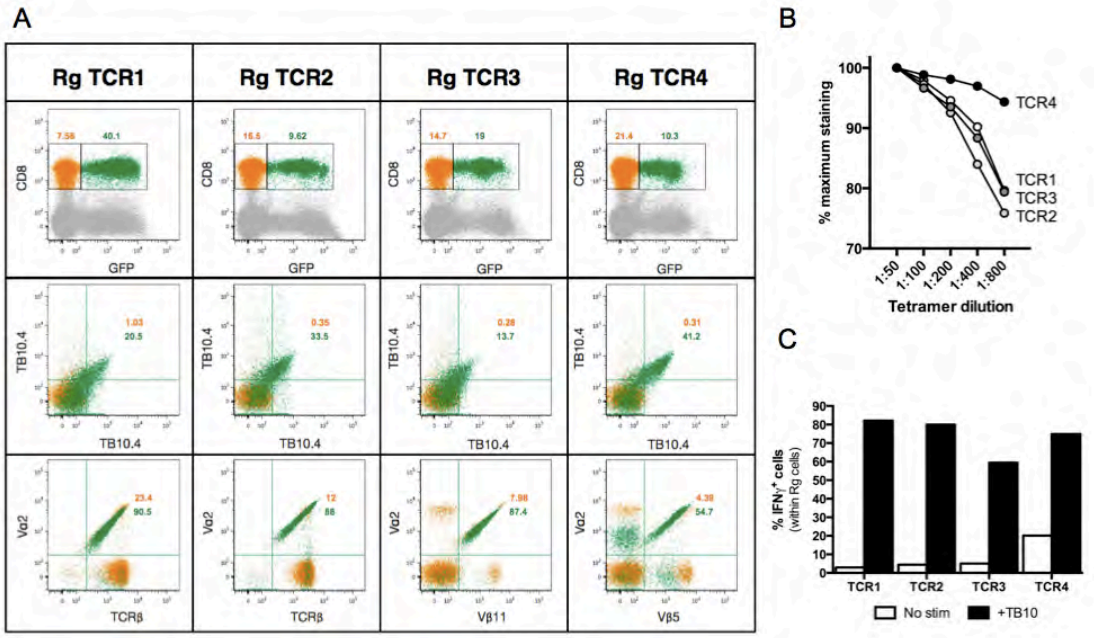

Figure 2: Retrogenic mice carry CD8$^+$ T cells that recognize and respond to TB10. Retrogenic mice have CD8$^+$ T cells that specifically recognize TB10, as they express GFP and stain with the respective Vα and Vβ antibodies, and bind the TB10 tetramer. CD8$^+$GFP$^+$ cells are colored in green, and control CD8$^+$GFP$^-$ cells in orange (A). The different TCRs recognize TB10 with different affinity/avidity, as measured by tetramer dilution (B). Retrogenic cells are specific for TB10, and produce IFNγ upon stimulation with their cognate peptide (C).

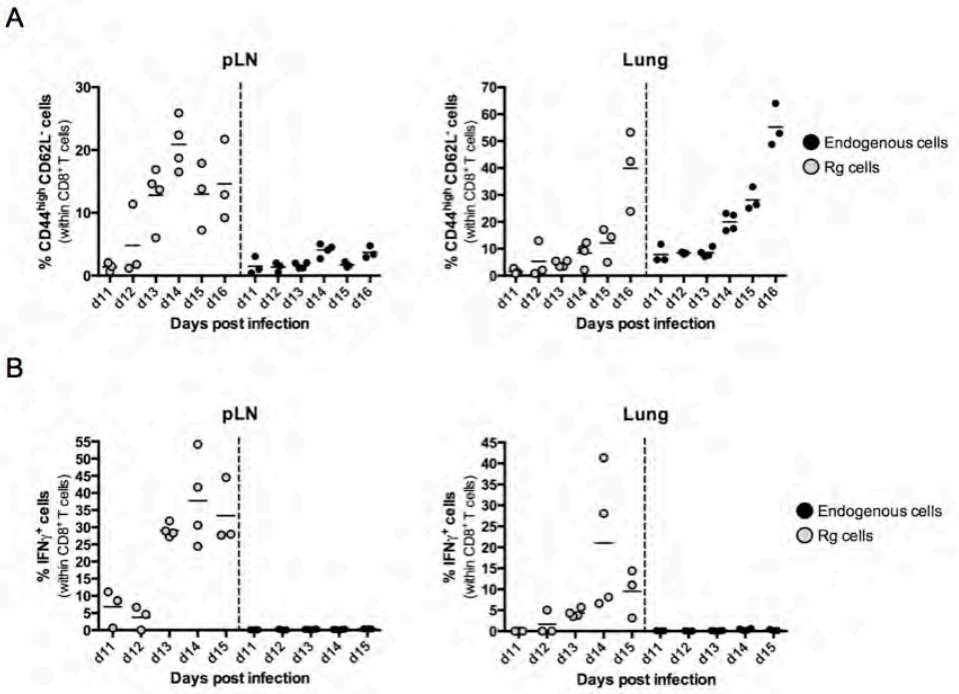

Figure 3: Retrogenic cells are primed between days 11 and 13, and rapidly acquire the ability to produce IFNγ. Naïve CD8$^+$ T cells from Rg mice were transferred into CD45.1 mice 1 day following aerosol infection, and their surface expression of CD44 and CD62L analysed in the dLN and lung between days 11 and 16 after aerosol infection (A). At the same time points, the ability of CD8$^+$ T cells to produce IFNγ was determined by intracellular cytokine staining after stimulation with the TB10 peptide (B).

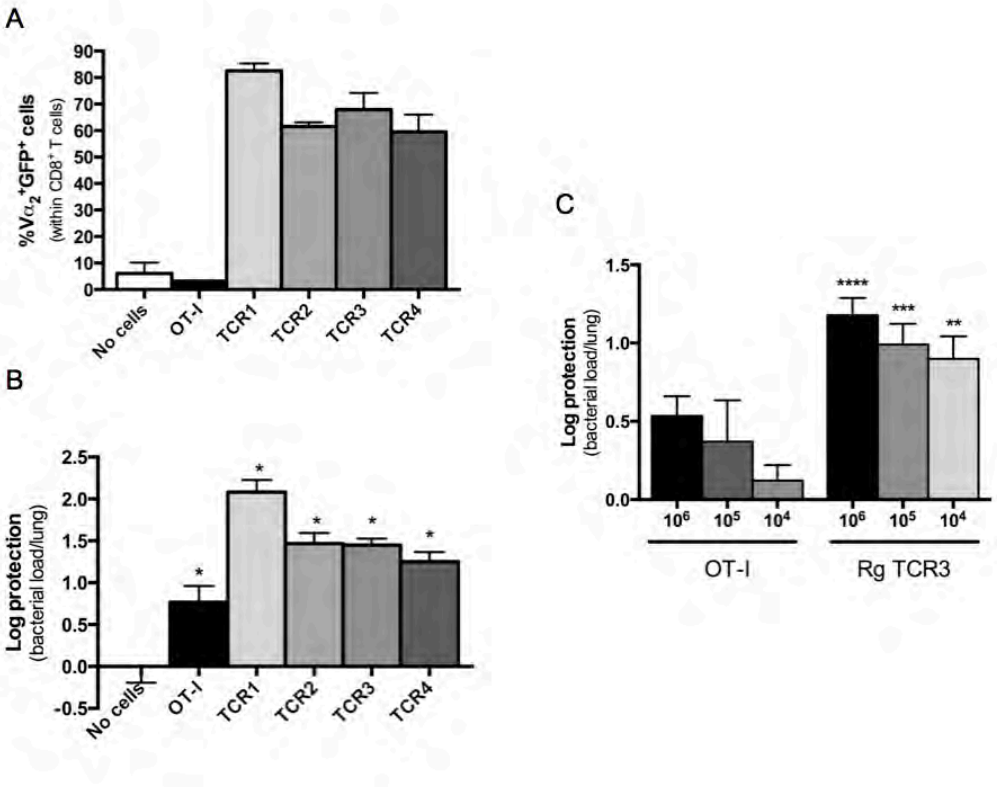

Figure 4: Retrogenic cells protect mice from aerosol infection. In vitro differentiated Rg cells or OT-I cells were transferred to aerosol infected sub-lethally irradiated B6 recipients, and their protective ability determined 21 days after transfer. Rg cells are present in the lung at day 21 as determined by GFP expression and staining with Vα2 antibody in CD8$^+$ T cells (A), and some Rg TCRs are capable of protecting mice, as assessed by bacterial burden in the lung at day 21 (B). Transfer of 10^4, 10^5 or 10^6 Rg cells confers protection against *M. tuberculosis* challenge, as assessed by bacterial burden in the lung at day 21 (C).

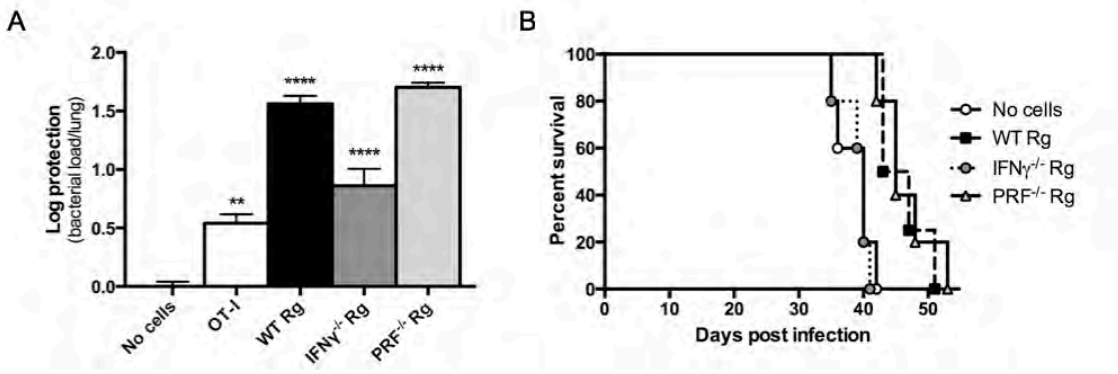

Figure 5: Protection mediated by retrogenic cells dependes on IFNγ production. In vitro differentiated Rg cells were transferred to sub-lethally irradiated B6 recipients (A) or naïve cells were transferred into TCR$\alpha^{-/-}$ mice (B), 1 day after aerosol infection with *M. tuberculosis.* The protective capacity of OT-I, WT, IFN$\gamma^{-/-}$ and Prf$^{-/-}$ cells was determined 21 days after transfer by measuring bacterial burden in the lung at day 21 (A) or by following survival (B).

Table 1: External and internal primers for single cell PCR

INTERNAL PRIMERS FOR TCRα CHAIN		INTERNAL PRIMERS FOR TCRβ CHAIN	
Name	**SEQUENCE**	**Name**	**SEQUENCE**
TRAV1_int	TGGATGGTTTGAAGGACAGTGGGC	VB1 Int	CTTTGGAATGTGAGCAACATC
TRAV2_int	CAAGGATATAAGGACTATGTGG	VB2 Int	CTTGAAGAATTCCCAGTATCCC
TRAV3_int	CGAAGGACAAGGATTCACTG	VB3 Int	CTTCAGCAAATAGACATGACTG
TRAV4_int	AACAAAGGAGAATGGRAGG	VB4 Int	CTTATGGACAATCAGACTGCC
TRAV5_int	GCAGATCCAAGGACTCATCG	VB5 Int	GCAGATTCTCAGTCCAACAG
TRAV6_int	CTCAGTGCATGAGTCAGACTC	VB6 Int	CAGGGAAAGGATTGAGACTG
TRAV6D-6_int	TGTTCGATAYCYTGGAGAAGG	VB7 Int	GAATGTGGACAGGACATGAG
TRAV7_int	TGGTACAGACAGCATYCTGG	VB8 Int	ATGTACTGGTATCGGCAGGAC
TRAV8_int	GTTCAAATGAGMGAGAGAAG	VB9 Int	GCAAGAGTTGGAAAACCAGTG
TRAV9 int	TGCTCCTCAAGTACTATTCVGG	VB10 Int	GTAAACGAAACAGTTCCAAGGC
TRAV10_int	GCTGGAAAGGGTCTCCACTTTGTG	VB11_Int	CAAGAAGCAACTCTGTGGTG
TRAV11_int	GTCAAATGGGAGATACTCAGC	VB12 Int	CTAAATTCATCCTTCTCCACTC
TRAV12 int	GCCACTCTCCATAAGAGCAG	VB13 Int	CTGTTTCCTTTTGGTGTGACC
TRAV13_int	CTTTGMMYATWTCCTCCTCC	VB14 Int	CTAACCTCTACTGGTACTGG
TRAV14_int	CACAATCTTCTTCAATAAAAGGG	VB15 Int	GAAGAACCATCTGTAAGAGTGG
TRAV15_int	GCCGCTATTCTGTAGTCTTC	VB16 Int	GTGACCCAGTTTCTAATCACC
TRAV16 int	CAGCAAGTGGGRAAATAGTTTT	VB17 Int	CCTGGTCAAAGAGAAAGGAC
TRAV17_int	CCAGAGCCTCCAGTTTCTCC	VB18 Int	GCTGACAGTCAAGTTGTTTCG
TRAV19_int	CACACTCCTGATATCCGTAC	VB19 Int	CTACAAGAAACCGGGAGAAG
TRAV20_int	AGAAGGGAAGATTCGAGGTG		
TRAV21_int	GTGACTCACGGTCTACAACAAAA		

EXTERNAL PRIMERS FOR TCRα CHAIN		EXTERNAL PRIMERS FOR TCRβ CHAIN	
Name	**SEQUENCE**	**Name**	**SEQUENCE**
TRAV1_ext	AGCAACGTGAAGGCCAAGCC	VB1 Ext	GGAAACAGCACTCATGAACAC
TRAV2_ext	CAAGAAGTGAACGTGTCCTG	VB2 Ext	CTACAGACCCCACAGTGAC
TRAV3_ext	TATCATYTGCACCTACACAGAC	VB3 Ext	CAAGATATCTGGTGAAAGGGC
TRAV4_ext	CTGCTCTGAGATGCAATTTTWC	VB4 Ext	AGTATCTAGGCCACAATGC
TRAV5_ext	TGGTATAAGCAAGAACCTGG	VB5 Ext	CTCYTGGGAACAAGTTCAGC
TRAV6 ext	TATCCCGGAGAAGGTCCACAGCTC	VB6 Ext	CACATGGTGATGGTGGCATC
TRAV6D-6_ext	GCACGTATTCAGCCACAAGCATAGG	VB7 Ext	GAACAGGCCTTGTGGACATG
TRAV7_ext	AGCAGAGCCCAGAATCCCTC	VB8 Ext	TGKGWRCAAAACACATGGAGGC
TRAV8_ext	CAGTGGTACAGACAGAAGTCAG	VB9 Ext	TGCAGCCACTTTTGTGGATAC
TRAV9 ext	AGCTGAGATGCAASTATTCCT	VB10 Ext	AATTGCTGAAGATTATGTTTAGC
TRAV10_ext	GTGTTCGAGAGGGAGACAGCGC	VB11 Ext	GAGAGCAGAACCAACAAATGC
TRAV11_ext	GGTGGTTCAAACAGGACAC	VB12 Ext	GCAAGTCTCTTATGGAAGATGG
TRAV12 ext	CTGTGATGCTGAACTGCACC	VB13 Ext	GTTCCTTGACACAGTACTGTC
TRAV13_ext	CAGTGGTTTTACCAAMRTCCT	VB14 Ext	GCTCAGACTATCCATCAATGG
TRAV14_ext	CCCAATCTCTGACAGTCTGG	VB15 Ext	TTCTGGGGCCTGGCTGTG
TRAV15_ext	GTGATTCAGGTCTGGTCAAC	VB16 Ext	GAAGCAGGACACACAGGAC
TRAV16_ext	GAAGACAACGGTGACAATGG	VB17 Ext	GCAGCTCTTTATGTTGCTGG
TRAV17_ext	CATACAGTGCAGCACCTTACC	VB18 Ext	TGTCCTCCTCTACCAAAAGC
TRAV19_ext	GATATGTTTGACTATTTTGCCTG	VB19 Ext	CTCTGGGGTTGTCCAGAATC
TRAV20_ext	TCACGCTCCTAATAGACATTC		
TRAV21_ext	CCAGATTCAATGGAAAGTACTG		

CONSTANT PRIMERS FOR TCRα AND TCRβ CHAINS			
TRACi	CGGCACATTGATTTGGGAGTC	TRBCi	AAGCCCCTGGCCAAGCACAC
TRACe	GGCCCCATTGCTCTTGGAATC	TRBCe	CTATAATTGCTCTCCTTGTAGG

Table 2: Primers for TCR amplification

	NAME	Sequence (all 5'-3')
TCR1β	RG1	CAACACTGAAGATGTTACTGC
	RG2	**GTTCCTGAGCCAAAATACAGCGTTT**CTCCCCCTGTCCGTCGATCCCTAGCACCACAGAGATATAAGC
	RG3	**AAACGCTGTATTTTGGCTCAGGAAC**CAGACTGACTGTTCTCGAGGATCTGAGAAATGTGACTCCAC
	RG4	CATCTTCACATCTGACTTCATG
TCR2β	RG5	TGACCCTACTATGGATATCTGG
	RG6	**AGTGCCTGGCCCAAAGTACTGGGTG**TCGCCCCAGTCCCGAGCGCTGCTGGCACAGAAGTACAC
	RG7	**CACCCAGTACTTTGGGCCAGGCACT**CGGCTCCTCGTGTTAGAGGATCTGAGAAATGTGACTCCAC
	RG8	ATCTTCACATCTGGCTTCATG
TCR3β	RG9	TTCTATGAGTGAAGCCACTGC
	RG10	**GTGCCCGCACCAAAGTACAAGGTGT**TTTGAACCCTGTCCCTTAAGCTGCTTGCACAAAGATACACC
	RG11	**ACACCTTGTACTTTGGTGCGGGCAC**CCGACTATCGGTGCTAGAGGATCTGAGAAATGTGACTCCAC
	RG12	ATCTTCACATCTGGCTTCATG
TCR4β	RG13	CCTGAGAAGAAGCATGTCTAAC
	RG14	**TGCCGGGACCGAAGTACTGTTCAAC**CCCCCCAGGCCTGAGAGAGCTGGCACAGAAGTACATAGCAG
	RG15	**GTTGAACAGTACTTCGGTCCCGGCA**CCAGGCTCACGGTTTTAGAGGATCTGAGAAATGTGACTCCAC
	RG16	ATCTTCACATCTGGCTTCATG
TCR1α	RG17	AGCTTCAGTCTAGGAAGAATGG
	RG18	**GGTCCCTTCCCCGAATGTTAATTTA**CCACTTAGTCCTCCAGTATCTGCACAGAAGTAGGTAGCTGAG
	RG19	**TAAATTAACATTCGGGGAAGGGACC**CAAGTGACGGTAATATCTGACATCCAGAACCCAGAACCTG
	RG20	TCAGTCTTGCAGACTCAACTG
TCR2α		SAME AS RG17
	RG21	**CCTTCCCCGAATGTTAATTTACCAC**TTAGTCCTCCAGTTCCCCGACTTGCTGCACAGAAGTAGGTAGCTGAG
	RG22	**GTGGTAAATTAACATTCGGGGAAGG**GACCCAAGTGACGGTAATATCTGACATCCAGAACCCAGAACCTG
		SAME AS RG20
TCR3α		SAME AS RG17
	RG23	**GGTCCCAGAGCCCCAGATCAGCTGA**TAGTTGTCACCCGTGCTGAGACAGAAGTAGGTAGCTGAGTCTCC
	RG24	**TCAGCTGATCTGGGGCTCTGGGACC**AAGCTAATTATAAAGCCAGACATCCAGAACCCAGAACCTG
		SAME AS RG20
TCR4α		SAME AS RG17
	RG25	**GGTCCCAGAGCCCCAGATCAGCTGA**TAGTTGTCACCTTCCCTTGCTGCACAGAAGTAGGTAGCTGAG
	RG26	**TCAGCTGATCTGGGGCTCTGGGACC**AAGCTAATTATAAAGCCAGACATCCAGAACCCAGAACCTG
		SAME AS RG20

Table 3: Primers for merge of TCR chains with 2A peptide

MOUSE		Sequence (all 5'-3')
TCR1	RG27	GCGCCAGAATTCAGATCTACC**ATGGACAAGATCCTGACAGC**
	RG28	CTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGAGCC**GGACCACAGCCTCAGC**
	RG29	CTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCC**ATGTTACTGCTTCTATTACTTCTGGG**
	RG30	GCGTCGCAATTGCTCGAG**TCATGAATTCTTTCTTTTGACCATAG**
TCR2	RG31	GCGCCAGAATTCAGATCTACC**ATGGACAAGATCCTGACAGC**
	RG32	CTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGAGCC**GGACCACAGCCTCAGC**
	RG33	CTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCC**ATGGATATCTGGCTTCTAGGTTG**
	RG34	GCGTCGCAATTGCTCGAG**TCATGAATTCTTTCTTTTGACCATAG**
TCR3	RG35	GCGCCAGAATTCAGATCTACC**ATGGACAAGATCCTGACAGC**
	RG36	CTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGAGCC**GGACCACAGCCTCAGC**
	RG37	CTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCC**ATGGCCCCCAGGC**
	RG38	GCGTCGCAATTGCTCGAG**TCATGAATTCTTTCTTTTGACCATAG**
TCR4	RG39	GCGCCAGAATTCAGATCTACC**ATGGACAAGATCCTGACAGC**
	RG40	CTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCTCCGGAGCC**GGACCACAGCCTCAGC**
	RG41	CTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCC**ATGTCTAACACTGTCCTCGCTG**
	RG42	GCGTCGCAATTGCTCGAG**TCATGAATTCTTTCTTTTGACCATAG**

Throughout this thesis we analyzed multiple aspects of immunity against mycobacterial infections. These included the description of the thymus as a place of ongoing immunity, the characterization of the impact of thymic infection of T cell differentiation, but also the role of $CD8^+$ T cells in peripheral immune responses. In this final chapter, we try to combine all these aspects into an overall model for T cell immunity to tuberculosis, and discuss the implications of our studies in the context of chronic infections, particularly in settings of co-infection with *M. tuberculosis* and HIV.

Chapter 6

Final discussion

An overall model for T cell responses: from the thymus to the periphery and back.

The data presented in this thesis addresses two distinct topics related to T cell immunity to mycobacteria. In the first part, we focus on the role of the thymus during infection. In the second, on how $CD8^+$ T cells contribute to immunity against *Mycobacterium tuberculosis* infection. We first review data on the thymus as a target of infection, and discuss how infection impairs thymic function. We then show how the thymus controls mycobacterial infections, by recruiting activated T cells from the periphery back to the thymus where they mount immune responses. In the second section, we explore immunodominance in order to generate a new mouse model to study when and how are $CD8^+$ T cells important in the immune response to tuberculosis. In combination, the results from these projects strengthen how important T cells are in controlling *M. tuberculosis* infection, and our studies clarify important aspects of T cell biology following infection. Taken together, these data allow us to propose a slightly revised model on the involvement of T cells during immunity to tuberculosis. This model will serve as the basis for this discussion section.

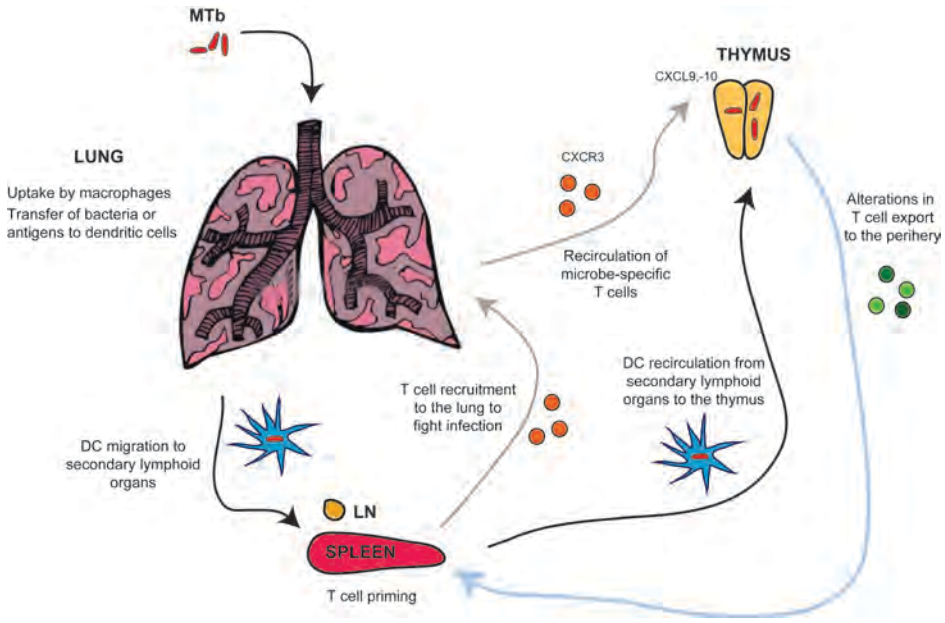

Figure 1. T cell responses following mycobacterial infection. Upon inhalation, lung macrophages uptake bacteria. Lung DC aquire bacteria and/or antigens, and transport them to the lymph node, where T cell priming is initiated. T cells are then recruited to the lung, to fight infection. We propose that DC recirculation from the secondary lymphoid organs to the thymus disseminates infection to this organ. To control infection, the thymus recruits activated T cells from the peripheral organs, in a process dependent on production of CXCL9 and CXCL10 by the thymus and the expression of CXCR3 by antigen-specific T cells. Infection of the thymus impairs thymic function and leads to alterations on the T cells exported from the thymus to the periphery.

Initiation of T cell responses

Tuberculosis usually starts with aerosol infection of the host, and bacterial uptake in the lung, mostly by resident macrophages[1]. Although T cell activation in the lung following infection has been described[2], most studies track initiation of adaptive immunity to the draining pulmonary lymph node (pLN), after *M. tuberculosis* antigens are carried to this organ by dendritic cells (DC)[3,4]. Our data strengthens this model, as we detect $CD8^+$ T cell priming by d12 after infection in the pLN, and activated $CD8^+$ T cells in the lung are only observed after this event (**Chapter 5**). How DC acquire such antigens, and how they are presented in the context of MHC-I molecules in order to prime $CD8^+$ T cells is still unclear. One possibility is that *M. tuberculosis* directly infects DC, and within these cells is either able to actively escape to the cytosol, or that *M. tuberculosis* proteins leak from the phagosome to the cytosol. This could be the result of active secretion pathways or through rupture of the phagosomal membrane. In either scenario, *M. tuberculosis* proteins would end up in the cytosol, where they could be processed and presented by MHC-I. A second possibility is that uninfected DC acquire *M. tuberculosis* antigens, either soluble or contained within vesicles, by phagocytosis, and transfer them from this exogenous sampling pathway to the cytosolic MHC-I pathway. This possibility, termed cross-presentation, as been described before, although the exact mechanisms behind it are still largely undefined[5]. The priming of $CD8^+$ T cells by cross-presentation is termed cross-priming. One way DC could acquire intracellular bacterial antigens is by efferocytosis, i.e., the uptake of apoptotic vesicles derived from infected macrophages. This is the basis for the “Detour model”, in which antigens present within apoptotic blebs from infected macrophages are taken up by DC and cross-presented to $CD8^+$ T cells[6,7]. This model is supported by our data, reporting that infected macrophages die by apoptosis in vivo, and are taken up by uninfected macrophages, in a process of efferocytosis that contributes to bacterial control[8]. Although the ability of DC to efferocytose apoptotic macrophages was not addressed in this study, we have previously shown that adoptive transfer of infected macrophages that are genetically prone to die by apoptosis leads to an earlier $CD8^+$ T cell response, in a process dependent on the present of DC[9]. In combination, these studies suggest that DC are able to engulf apoptotic vesicles derived from infected macrophages, process *M. tuberculosis* antigens, and prime $CD8^+$ T cells.

Another important question is why T cell priming is delayed following *M. tuberculosis* infection. Of note, *M. tuberculosis* infection has been reported to impair DC maturation[10], prevent up-regulation of MHC-II[11], and inhibit antigen presentation[12].

Since DC seem to be essential for the initiation of T cell responses in the pLN, these observations could explain why priming is delayed. Interestingly, T cell priming – observed between days 11 and 12 – only seems to occur after bacterial dissemination to the pLN – detected around day 8 after infection[3,4,13]. These data suggest that live bacteria within DC may be necessary for the initiation of T cell priming. However, these results also indicate that DC could help disseminate *M. tuberculosis* infection from the lung. Another possibility is that T cell priming is dependent on antigenic levels within the pLN; therefore, only after bacterial dissemination to this organ and active replication in the pLN would priming be initiated. Interestingly, our data using infected macrophages that are prone to die by apoptosis demonstrates that initiation of T cell priming can be accelerated, even in the context of lower bacterial loads[9], suggesting that the amount of antigen present in the pLN might not correlate with T cell priming efficiency. Alternatively, it is possible that T cell responses are geared towards bacterial antigens that are only expressed later during infection, and this is why T cell priming is a late phenomenon. This hypothesis is hard to test, since the kinetics of antigen expression by *M. tuberculosis* are still unclear, but such difference between "early" and "late" antigens could also explain why $CD4^+$ T cells seem to be primed a few days earlier than $CD8^+$ T cells – $CD4^+$ T cells specific for ESAT-6 are primed between days 7 and 10 [13], while $CD8^+$ T cells specific for TB10 are primed between days 11 and 13 (**Chapter 5**). Of note, some *M. tuberculosis* proteins (like TB10) contain both $CD4^+$ and $CD8^+$ T cell epitopes, a feature that could be used to dissect between differences in the timing of proteins expression versus intrinsic differences between the two T cell subsets. Finally, it is possible that a delay in antigen processing and transfer of antigens from the MHC-II to the MHC-I pathway only allows $CD8^+$ T cells to be primed after $CD4^+$ T cells. This is a hypothesis that could also be tested by using different $CD4^+$ and $CD8^+$ T cell epitopes from the same bacterial proteins.

The importance of immunodominance for $CD8^+$ T cell responses

Despite a slow start, once T cell priming is initiated, T cells expand rapidly and are fast to acquire effector functions, such as the ability to produce IFNγ (**Chapter 5**). Interestingly, T cell responses are incredibly focused to some *M. tuberculosis* antigens, in a process designated as immunodominance[14]. This is particularly true for $CD8^+$ T cell responses to TB10, in which 30-50% of the cells in the lung at the peak of infection are direct towards a single epitope[9,15,16]. Furthermore, within each individual host, TB10-specific cells are (oligo)clonal (**Chapter 5**). Why

this happens is still unclear. There are three factors that determine the magnitude of antigen-specific $CD8^+$ T cell responses: the precursor frequency of naïve T cells specific for a certain epitope, the capacity to recruit naïve T cells to the LN during priming and the subsequent expansion of the primed cells. Although we did not address how efficient naïve T cell recruitment is following *M. tuberculosis* infection, we have shown this process to be highly efficient during infection with other pathogens at multiple infectious doses[17]. Also, by comparing immunodominant responses to *M. tuberculosis* with subdominant responses, we have reported that naïve T cell precursor frequency does not correlate with the magnitude of the $CD8^+$ T cell response[15]. Furthermore, manipulation of the precursor frequency by the means of vaccination was not sufficient to alter immunodominance following infection[15]. These data suggest that *M. tuberculosis* infection, rather than host factors, drives the expansion of the immunodominant T cell clones. These observations are particularly relevant in the context of vaccination. The assumption for vaccine design has been that a good vaccine will be able to boost T cell numbers prior to infection, and that the T cells elicited by vaccination should be protective. However, in the case of tuberculosis, what constitutes a protective T cell response is still unclear. Although the dominant view is that IFNγ is essential for the protective capacity of T cells, IFNγ levels do not correlate with protection conferred by vaccination[18,19], i.e., IFNγ is necessary but not sufficient to protect the host. Also, since infection, and not the host, seems to determine the expansion of T cells, modulation of T cell precursor frequencies by vaccination may never result in protection against *M. tuberculosis* infection. Therefore, it will be important to understand what are the protective features of T cells and whether those can be elicited by vaccine strategies, and whether these can be expressed by immunodominant T cells. Finally, it is still unclear whether immunodominant T cell responses are beneficial for the host, or are a survival strategy employed by the bug to subvert immunity. Although it has been shown that T cell epitopes of *M. tuberculosis* antigens are evolutionary hyperconserved, suggesting that immune recognition is beneficial to the bug[20], our observation that TB10-specific $CD8^+$ T cells are protective during infection (**Chapter 5**) argues in favor of immunodominance being beneficial for the host. This notion is supported by other data on protective T cell responses mediated against immunodominant antigens, such as ESAT-6 and Ag85[3,13,21]. Furthermore, it is interesting to note that, although most *M. tuberculosis* antigens are hyperconserved, that is not the case for TB10, whose antigens display variability[20]. Therefore, although further studies are necessary to clarify this relationship, immunodominance

"per se" does not seem to preclude T cell protection, and therefore is probably not a bacterial subversion mechanism to evade adaptive immunity.

Protective mechanisms of T cell-mediated immunity

Once primed, T cells migrate to the lung, where they recognize infected cells and mount immune responses that lead to the control of bacterial growth. Interestingly, how T cells control infection is still largely unknown. Dogma is that production of pro-inflammatory cytokines by T cells, namely IFNγ and TNF, stimulates infected macrophages to produce reactive oxygen and nitrogen species with antibacterial potential, leading to bacteria control[22,23]. Our data that IFNγ production is essential for protection mediated by $CD8^+$ T cells (**Chapter 5**) is in agreement with this model, although the exact mechanism(s) by which IFNγ controls bacterial burden in this system needs further elucidation. This data is also in agreement with previous studies reporting that IFNγ production by $CD8^+$ T cells controls *M. tuberculosis* infection[24]. Additionally, we are now generating retrogenic mice lacking the ability to produce TNF in order to investigate the role of production of this cytokine in the protection mediated by $CD8^+$ T cells. However, it is now clear that IFNγ production by T cells does not correlate with protection, and that several IFNγ-independent mechanisms of *M. tuberculosis* control exist. This is best exemplified by studies using $CD4^+$ T cells specific for ESAT-6, in which the observed protection mediated by these cells does not require IFNγ or TNF production[25]. In a different study, IFNγ production by $CD4^+$ T cells did not improve bacterial control at early stages of infection, but was necessary for long-term control of bacterial replication and improved host survival[26]. Interestingly, IFNγ production by $CD4^+$ T cells was also essential for optimal $CD8^+$ T cell responses[26], and $CD4^+$ T cells have been shown to be essential for IFNγ production by $CD8^+$ T cells[27]. These results strengthen the notion that IFNγ is necessary for optimal immunity during tuberculosis, but that its function might be broader than just acting to activate infected macrophages. This notion is strengthen by the immunoregulatory role that IFNγ plays during infection, such as regulating neutrophils[28] or controlling IL-1β-driven immune pathology[29]. Furthermore, these studies demonstrate that other T cell functions, rather than IFNγ production, are essential for optimal immunity against *M. tuberculosis* infection. To address this question, we have started to perform microarray analysis of both $CD4^+$ and $CD8^+$ T cells sorted from the lung of infected

animals at different stages of *M. tuberculosis* infection (data not shown). We believe that these data, in combination with our retrogenic mouse system, will allow us to identify and target new pathways that might be essential for T cell mediated control of bacterial replication.

Importantly, the observations that $CD4^+$ T cells and their production of IFNγ modulate $CD8^+$ T cell function might also explain the discrepancy between the roles of these two T cell subsets during infection[26,27,30]. Historically, the observation that mice without $CD4^+$ T cells are more susceptible to tuberculosis than mice lacking $CD8^+$ T cells has been interpreted has proof that $CD4^+$ T cells are essential in this setting, while $CD8^+$ T cells are redundant[31]. However, if $CD4^+$ T cell help is essential for $CD8^+$ T cell function, an absence of $CD4^+$ T cells would imply a defect of the activity of both subsets. This possibility remains to be addressed experimentally, but is supported by the observation that antibody depletion of both $CD4^+$ and $CD8^+$ T cells is more detrimental than depletion of $CD4^+$ T cells alone[31]. How $CD4^+$ T cells regulate $CD8^+$ T cell function is also a matter of debate. One possibility is that IL-2 production by $CD4^+$ T cells is necessary for $CD8^+$ T cells to acquire the ability to be cytolytic[32]. We are currently addressing this possibility, as it could explain why in our experimental system TB10-specific Rg cells do not require perforin for protection (**Chapter 5**), while others have reported that perforin is essential for $CD8^+$ T cell mediated killing and protection in vivo[16]. This apparent contradiction might be explained by the source of the $CD8^+$ T cells. In our study, Rg cells specific to a single *M. tuberculosis* epitope are differentiate into T_c1 effectors by short term in vitro culture. In contrast, Woodworth et al. used polyclonal $CD8^+$ T cells purified from the spleen of infected animals[16]. These cells differentiated in vivo for a long period of time (over 5 weeks), in the presence of $CD4^+$ T cells, which could account for the observed differences. Of note, IL-2 treatment of $CD8^+$ T cells has been shown to upregulate the expression of perforin, but only after more than 10 days in culture[33]. This could explain why CTL capacity only emerges at later stages of infection, and why this is not mimicked by our short-term culture conditions. We are currently addressing these possibilities, namely the capacity of our Rg cells to differentiate into CTLs in the presence of IL-2 and/or $CD4^+$ T cells, when does such differentiation occur. These experiments should provide valuable insight on the generation of CTLs in vivo during tuberculosis and their contribution to $CD8^+$ T cell mediated protection.

Bacterial dissemination to the thymus

Although T cells are recruited to the lung and are able to control bacterial replication, they fail to eradicate infection and *M. tuberculosis* persists in the host for extended periods of time[34]. This ability to persist may contribute to *M. tuberculosis* dissemination to other organs, a characteristic of mycobacterial infections. This is particularly true for lymphoid organs, and *M. tuberculosis* is commonly found in the spleen and lymph nodes. More interestingly, primary lymphoid organs are also infected, namely the bone marrow[35] and the thymus[36]. Because the thymus is the place where T cells differentiate[37], and since T cells play an essential role during mycobacterial infections, we investigated the role of thymic infection following mycobacterial challenge.

Previous studies from our laboratory described the thymus as a target of mycobacterial infection, with different mycobacterial species and different infectious routes leading to thymic infection[36]. Interestingly, dissemination to the thymus occurs later than to other organs, but follows the same pattern as in other tissues: the initial bacterial burden is low, rises sharply for the first weeks after infection, and eventually plateaus; bacterial clearance is never achieved. How thymic infection is initiated is unclear, but several scenarios are possible. The first possibility is that free bacteria make it to the blood, enter the thymus and infect thymic cells either in a targeted or a random manner. Although this is possible, *M. tuberculosis* is a facultative intracellular pathogen, is mostly found within cells, and has never been reported freely in the blood. A more plausible explanation is that *M. tuberculosis* travels to the thymus within cells. We now appreciate that several cell types traffic between the thymus and the periphery. This is true for both peripheral T cells[38-40], and specific DC subsets (such as Sirpα$^+$ cDCs)[41-43], that migrate from the periphery to the thymus, where they modulate thymic function. If infected, these cells could seed infection in the thymus. This “Trojan horse” model is supported by the observation that DC are responsible for disseminating *M. tuberculosis* from the lung to the draining lymph node[3], raising the possibility that dissemination to the thymus can occur by a similar mechanism. Furthermore, we have shown that mycobacteria reside within macrophage and DC-like cells within the thymus[44]. This model could also explain why thymic infection starts later than in other organs; *M. tuberculosis* dissemination to secondary lymphoid organs could precede DC recirculation to the thymus and delay the start of thymic infection. This could also be true if thymic infection is dependent on the dissemination of infected cells from the bone marrow to the thymus, as bone marrow infection is also only observed at later stages of infection[35].

Consequences of thymic infection

T cells differentiate in the thymus, a process dependent on thymic architecture, the antigens encountered by thymocytes, and the milieu surrounding the differentiating cells[37]. Because the thymus is a target of mycobacterial infection, and since infection can alter these features, infection can interfere with thymic function. In fact, our previous studies addressed how infection impacts thymic development. To do so, we infected mice with a *M. avium* strain of intermediate virulence that leads to chronic infection without inducing premature thymic atrophy[44,45]. Following infection, the thymus continues to support T cell differentiation, but the T cells that differentiate in this environment are abnormal; T cells that differentiate within infected thymi are suboptimally activated by *M. avium* antigens when compared to T cells that differentiate in uninfected thymi[44]. This defect is specific for *M. avium* antigens, as T cells from both groups respond similarly to model antigens[44]. These data indicate that infection of the thymus can induce pathogen-specific T cell tolerance. Our observations are in agreement with studies on tolerance induction in viral models, such as following MLV, LCMV and HBV infection (reviewed in **Chapter 2**)[46-48]. It will be important to determine what is the mechanism of tolerance induced by thymic infection. The possibilities are the generation of pathogen-specific regulatory T cells, the induction of T cell anergy on the developing T cells or negative selection of differentiating pathogen-reactive T cells. These possibilities are currently being investigated, and this is an example of how the studies in the two sections of this thesis could be connected, as the use of retrogenic mice is expected to contribute to elucidate these questions.

Thymic control of mycobacterial growth

The observation that bacterial growth is halted in the thymus at later stages of infection suggests that an immune response is established in this organ. In fact, we detect antigen-specific $CD4^+$ and $CD8^+$ T cells within the thymus following *M. avium* and *M. tuberculosis* infection (**Chapter 3**)[49]. These T cells are able to produce pro-inflammatory cytokines and activate infected cells, specifically by stimulating the production of inducible nitric oxide synthase (iNOS), contributing to bacterial control in this organ (**Chapter 3**)[49]. Importantly, we also addressed the origin of the responding T cells and demonstrated that these are not newly differentiated mature

thymocytes but instead activated peripheral T cells that recirculate back from peripheral organs to the thymus to fight infection (**Chapter 3**)[49]. The recruitment of activated T cells is associated with increased expression of T helper 1 chemokines in the infected thymus and an enrichment of $CXCR3^+$ mycobacteria-specific T cells within this organ (**Chapter 3**)[49]. These results suggest that T cell recirculation from the periphery to the thymus is a mechanism that helps the immune system respond to thymic infection. These data are supported by other studies that also detected antigen-specific T cell immunity in the thymus following infection with *M. tuberculosis*[50] and other pathogens. This is the case for LCMV, in which adoptively transferred LCMV-specific CTL traffic to the thymus and eliminate the virus from this organ[47,51]. Similarly, during influenza infection, functional influenza-specific CTLs are detected in thymi from HPAIV infected mice[52]. These results confirm that the thymus is not only a site of infection, but also a place of ongoing immunity, and that T cell recirculation from peripheral organs back to the thymus contributes to protect the thymus from infectious agents.

Relevance of thymic infection during mycobacterial infection

Taken together, our studies challenge the notion that the thymus is irrelevant during infection, as we show that the thymus is a place of infection and ongoing immunity, and that thymic infection has the potential to alter the peripheral T cell pool and impact ongoing immune responses. We believe these data to be relevant at least in two different scenarios. The first is in infants, when the T cell repertoire is still being formed. In this case, mycobacterial presence in the thymus could impact not only short-term immunity, but also have long lasting consequences for future immune response. Interestingly, BCG also disseminates to the thymus[36], and this observation should be explored in the context of administration of live vaccines, as their presence and replication within the thymus could lead to tolerization of differentiating T cells.

The impact of thymic infection during adulthood could also be of relevance. The argument against this is the prediction that the peripheral T cell pool should include pre-existing pathogen-specific T cells capable of fighting infection. However, in the context of persistent infection – such as tuberculosis – coupled with severe lymphopenia – such as during HIV – the emergence of tolerance to the infectious agent may impair deployment of pathogen-reactive T cells in the naïve repertoire. This would prevent the replenish of antigen-specific T cells in the peripheral T cell pool, and favor the microbe in the long-term, since it would impair T cell immunity

and contribute to pathogen persistence within the host. This possibility should be addressed experimentally in future studies focusing on TB/HIV co-infections.

Future research

The data presented and discussed in this thesis contributes to a better understanding of the role of T cells during mycobacterial infections. However, several important aspects of T cell biology remain unclear. Here I try to summarize what I consider the most important issues to address in future studies, with the hope that answering such questions will provide new insight into how T cells control *M. tuberculosis* infection and contribute in the global fight against tuberculosis.

The role of the thymus during infection

How universal is thymic infection? We show that mycobacteria disseminate to the thymus, and review data on the ability of multiple viruses, bacteria, fungi and parasites to infect the thymus, but is this a universal feature of microbes or specific for certain pathogens? These studies should also focus on different strains of the same pathogen, since strain-specific differences in the ability to infect the thymus could provide insight into the mechanisms of thymic infection. Also, studies on human populations would address the ultimate significance of these findings.

How does the thymus get infected? The “Trojan-horse” model postulates that cell recirculation from the periphery to the thymus initiates thymic infection, but is this true for all pathogens or are there any other mechanisms that lead to infection of the thymus? One way this question could be addressed is by adoptive transfer of sorted cells infected with reporter strains of pathogens (such as fluorescent bacteria). Studies depleting specific cell subsets (such as the recirculating subset of DC that expresses Sirpα) also have the potential to be very informative.

How does thymic infection lead to T cell tolerance? What is the mechanism? Does infection of the thymus lead to the development of pathogen-specific regulatory T cells that dampen immune responses? Is the presence of microbial antigens within the thymus sufficient to induce negative selection of pathogen-specific T cells? Such studies can greatly benefit from the retrogenic technology described in this thesis, as bone marrow cells with the potential to

differentiate into mycobacterial-specific T cells can be transferred to mice with infected thymi, and their ability to give rise to mature T cells or Tr_{egs} can be assessed.

How does thymic infection during childhood affect future T cell immunity? The observation that thymic infection leads to the generation of pathogen-specific tolerance should be addressed in the context of natural infections, but also during vaccination.

How does the thymus control infection? We propose a model in which activated T cells recirculate from the periphery back to the thymus to fight infection, in a CXCR3-dependent manner. Is this specific for mycobacteria, or are there other ways to recruit immune T cells to the thymus to fight infection? Are there any innate mechanisms that prevent thymic infection? These questions can be addressed with the same approaches that have been used in studying immunity in other organs; the biggest challenge here is conceptual, since the thymus should be considered an organ of active disease.

The role of $CD8^+$ T cells during infection

What constitutes protective immunity to tuberculosis? $CD4^+$ T cells and IFNγ are both necessary for a successful immune response against *M. tuberculosis*, but clearly not sufficient. What other mechanisms contribute to bacterial control? These studies will benefit from our gene expression profiling analysis, and the ability to select new targets that can be validated by the use of retrogenic mice.

How are $CD8^+$ T cells protective during *M. tuberculosis* infection? Our data supports that IFNγ production by these cells contributes to protection, but is this the only thing they do? When are $CD8^+$ T cells important? What is more important, cytokines or cytotoxicity? The establishment of in vitro systems to differentiate naïve Rg $CD8^+$ T cells into different phenotypes should allow adoptive transfer experiments similar to the ones presented in this thesis to clarify these questions.

How do $CD4^+$ T cells modulate $CD8^+$ function? Is $CD4^+$ T cell help necessary for the emergence of functional CTL? Is this dependent on IL-2? This could be addressed by a combination of in vitro strategies to differentiate $CD8^+$ T cells in the presence or absence of IL-2 and adoptive transfer experiments of Rg cells with or without co-transfer of $CD4^+$ T cells.

Why are $CD8^+$ T cell responses to *M. tuberculosis* immunodominant? Are immunodominant epitopes protective? Is immunodominance a bacterial strategy

to evade host immunity? Should vaccination focus on immunodominant epitopes, or subdominant ones? Does TCR affinity dictate immunodominance and the protective capacity of T cells? These studies will benefit from our establishment of different Rg mouse models to both immunodominant and sub-dominant epitopes, with different affinities to their cognate peptides.

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