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INFECTION OF THE THYMUS BY MYCOBACTERIA: POTENTIAL IMPACT ON THE IMMUNE RESPONSE TO INFECTION

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ABBREVIATION LIST

AFB	Acid fast bacilli
Ag85	Antigen 85
AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacillus Calmete-Guérin
BTB	Blood-thymus barrier
CD	Cluster of differentiation
CD4 SP	CD4 single positive ($CD4^+CD8^-$)
CD8 SP	CD8 single positive ($CD4^-CD8^+$)
CFU	Colony forming units
CLA	Clarithromycin
Con A	Concanavalin A
CR	Complement receptor
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3-grabbing non-integrin
DN	Double negative ($CD4^-CD8^-$)
DP	Double positive ($CD4^+CD8^+$)
D- value	Decimal reduction time
EMB	Ethambutol
Foxp3	Forkhead box P3
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase

i.v.	Intravenous
KO	Knock-out
LAM	Lipoarabinomannan
LN	Lymph node
LPS	Lipopolysaccharide
MAC	*Mycobacterium avium* Complex
MHC	Major Histocompatibility Complex
NK	Natural killer
OVA	Ovalbumine
PCR	Polymerase chain reaction
PPD	Purified protein derivative test
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RAG	Recombination activating gene
RFP	Rifampicin
RNS	Reactive nitrogen species
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TREC	T cell receptor excision circles
TSA	Tissue-specific antigen
WHO	World Health Organization
WT	Wild-type

ABSTRACT

Understanding the immune response against bacteria of the genus *Mycobacterium* is fundamental, not only given the number of people infected with *Mycobacterium tuberculosis* (estimated to be about one third of the world's population) but also due to the vast number of sick individuals and deaths caused by infection with these bacteria and *Mycobacterium avium*. The rise of mycobacterial infections in immune-compromised patients, especially in individuals co-infected with the human immunodeficiency virus (HIV), highlighted the importance of $CD4^+$ T cells in the control of mycobacterial infections. At the time this project started, we had preliminary data showing that in *M. avium* infected mice, bacteria disseminate to the thymus. Since T cell differentiation occurs mostly within the thymus, this initial observation prompted us to further characterize thymic infection by mycobacteria and how infection of this organ influences T cell differentiation and the host immune response against mycobacteria.

We show here that the thymus is a target organ for *M. avium* or *Mycobacterium bovis* bacillus Calmete-Guérin (BCG), upon intravenous infection, and for *M. tuberculosis* upon aerogenic infection. Bacterial growth lasted longer in the thymus than in organs such as the spleen and the liver, suggesting that the host's immune response against mycobacteria is weaker and/or delayed within the thymus than in other organs. Of notice, antibiotic treatment with doses able to eliminate *M. avium* in the spleen, lung and liver, was much less efficient against the *bacilli* in the thymus. This may be of great concern, since when chemotherapy is usually considered completed, after a period of 6 to 12 months, bacteria may still reside within the thymus.

Histological analysis of infected thymi revealed that bacteria are mainly localized in large clumps of dendritic cells and macrophages at the medulla and cortico-medullary regions. Infection of the thymus does not result in premature thymic atrophy, as observed in other models of infection with thymic dissemination. Even though the ability to generate new T cells is retained by mycobacteria-infected thymi, T cells that differentiate in these circumstances were not able to protect mice against *M. avium* infections; yet, the ability to respond to unrelated antigens upon immunization was maintained.

The work developed in this thesis provides insight that might be of relevance to understand the repertoire of T cells generated during T cell reconstitution of lymphopenic individuals, such as patients with acquired immunodeficiency syndrome (AIDS) following anti-retroviral therapy, and with a mycobacterial co-infection. Moreover, it highlights the need to consider the thymus in studies addressing new therapeutic strategies against mycobacterial infections. Future studies should address the mechanisms responsible for

the induced tolerance to mycobacteria in infected thymi, and the mechanisms underlying the decreased ability of antibiotics to eliminate bacteria within the thymus.

RESUMO

O estudo da resposta imunitária do hospedeiro à infecção por bactérias do género *Mycobacterium* é essencial não só por haver um grande número de pessoas infectadas com *Mycobacterium tuberculosis* (estima-se que corresponda a cerca de um terço da população mundial) como também pela morbilidade e mortalidade associada às infecções por esta bactéria e por *Mycobacterium avium* serem bastante elevadas. O aumento de casos de infecções com micobactérias em indivíduos imunocomprometidos, especialmente em situações de co-infecção pelo vírus da imunodeficiência humana, realçou a importância das células T $CD4^+$ no controlo das infecções micobacterianas. No momento em que este projecto se iniciou, dados preliminares do nosso laboratório mostravam que em infecções com *M. avium* havia disseminação de bactérias para o timo. Uma vez que a diferenciação de células T ocorre maioritariamente no timo, esta observação despoletou o interesse na caracterização da infecção do timo com micobactérias e no estudo do impacto da infecção do timo na diferenciação de linfócitos T e da resposta imunitária do hospedeiro às micobactérias.

Neste trabalho demonstrámos que a disseminação de bactérias para o timo é observada após infecção intravenosa com *M. avium* ou com *Mycobacterium bovis* bacillus Calmete-Guérin (BCG) e também após inalação de aerossóis contendo *M. tuberculosis.* A infecção no timo decorre de uma forma progressiva sendo o *plateau* bacteriano atingido mais tarde do que noutros órgãos, o que sugere que a resposta imunitária no timo é mais fraca e/ou que demora mais tempo a estabelecer-se. É importante salientar que a administração de antibióticos capazes de reduzir a carga bacteriana no baço, fígado e pulmão, é menos eficaz a eliminar os bacilos contidos no timo. Esta observação é de extrema relevância já que sugere a possibilidade de haver persistência de bactérias no timo após o término do tratamento antibiótico o que, no caso dos humanos, varia entre 6 a 12 meses.

A análise histológica de timos infectados revelou que as bactérias estão principalmente localizadas em aglomerados de células dendríticas e macrofágos na medula e zona cortico-medular. No entanto, e ao contrário do que acontece noutras infecções em que o timo é também um órgão alvo, no nosso caso não se observa atrofia prematura do timo. É ainda importante salientar que apesar da produção de células T pelo timo infectado não estar comprometida, essas células não são capazes de estabelecer uma resposta imunitária eficiente durante uma infecção com *M. avium.* No entanto, a capacidade de responder a antigénios distintos dos micobactérianos não parece estar alterada.

Os resultados obtidos no âmbito deste trabalho poderão ajudar a compreender melhor o repertório de células T gerado em situações de reconstituição imunológica,

especialmente em pacientes com síndrome de imunodeficiência adquirida em tratamento anti-retroviral e com uma co-infecção micobacteriana. Estes dados destacam também a necessidade de considerar o timo quando novas estratégias terapêuticas contra infecções micobacterianas são exploradas. Estudos futuros são necessários para determinar os mecanismos responsáveis pela indução de tolerância às micobactérias das novas células T geradas num timo infectado, bem como os factores inerentes à capacidade diminuída dos antibióticos em eliminar micobactérias no timo.

RESUMÉ

Les personnes sont, partout dans le monde, fréquemment exposées à des bactéries du genre *Mycobacterium*. Les infections causées par des mycobactéries sont devenues de plus en plus importantes ces dernières années, à cause de l'énorme numéro d'individus infectés par *Mycobacterium tuberculosis* (à peu près un tiers de la population mondiale) et du grand numéro de malades et de morts dû à des infections par cette bactérie et par *Mycobacterium avium*. Le grand numéro de cas de coïnfection HIV-mycobactéries a renforcé l'importance des cellules T $CD4^+$ pour le contrôle des infections causées par des mycobactéries. Au moment ou ce projet commençait, on venait de découvrir au laboratoire que dans des souris infectées par *M. avium* le thymus devient colonisé par ces bactéries. Cette découverte a déclenché une nouvelle ligne de recherche pour caractériser l'infection du thymus par les mycobactéries et pour élucider es conséquences sur la différentiation des lymphocytes T et la réponse immunitaire contre les mycobactéries.

Nous avons démontré que le thymus devient colonisé par des mycobactéries quand les souris sons infectées par *M. avium* et *Mycobacterium bovis* Bacillus Calmete-Guérin (BCG) par voie intraveineuse, et aussi quand les animaux sont infectés par l'inhalation des aérosols contenant *M. tuberculosis*. La colonisation du thymus par les mycobactéries survient progressivement et l'arrêt de la croissance du numéro de bactéries dans le thymus parvient quelques semaines plus tard que dans d'autres organes, tel que la rate et le foie. Cette observation suggère que dans le thymus la réponse immunitaire est établie plus tard ou qu'elle est moins efficace. Nous avons observé que pendant un protocole de traitement antibiotique qui élimine les bactéries dans le foie et la rate, les bactéries dans le thymus ne sont pas éliminées. Ces résultats montrent que les bactéries dans le thymus sont moins sensibles aux traitements antibiotiques que les bactéries dans d'autres organes, et suggère qu'après avoir terminé un traitement antibiotique il est possible que des bactéries viables restent encore dans le thymus.

Des études de l'histologie du thymus ont démontré que les bactéries se retrouvent surtout dans des agrégés de macrophages e de cellules dendritiques dans la région cortico-medullaire e dans la médulle du thymus. En étudiant le thymus de souris infectées, comparées avec des non infectées, nous avons découvert qu'il n'y a pas des différences significatives dans le numéro de cellules; nos résultats suggèrent que la production de nouveaux lymphocytes est maintenue malgré l'infection. Nonobstant, les cellules qui se différencient dans des thymus infectés ne sont pas capables d'établir une réponse immunitaire efficace contre les mycobactéries; par contre ces cellules son capables de répondre à d'autres stimuli non-mycobacteriens.

Les résultats obtenus au sein de cette thèse sont probablement très importants pour meilleur comprendre le répertoire de lymphocytes T dans des situations de reconstitution immunologique de patients avec le syndrome de l'immunodéficience acquise, pendent leur traitement antirétroviral, et s'il y a une coïnfection mycobactérienne. Nos observations suggèrent aussi que le thymus doit être considéré pendant la recherche de nouveaux traitements contre les infections mycobactériennes. Des travaux futurs pourront élucider les mécanismes responsables par la production de lymphocytes T tolérants aux antigènes de mycobactéries par des thymus infectés, et aussi les mécanismes responsables par la réduction de l'efficacité des antibiotiques sur des bactéries dans le thymus.

THESIS OUTLINE

The present dissertation is organized in five chapters. In chapters 2, 3 and 4 the results obtained throughout this thesis are presented in the form of article.

In chapter 1, a general introduction to the subject of this thesis is presented. An overview is made on several aspects of mycobacterial infections such as the relevance of the disease for the humankind; significant microbiological aspects of the bacteria from the *Mycobacterium* genus; the recognition of the *bacilli* by the host; and the innate and acquired immune response to mycobacterial infections, focusing mainly in the role of T cells. Next, a glance through the function of the thymus as an organ where most T cells differentiate is made and the role of this organ in the adulthood is reviewed. Finally, an overview throughout the models of thymic infection and on published human case-reports of mycobacterial thymic infection is made. At the end of this chapter, the aims of this thesis are presented.

Chapter 2 is composed of a report, published in *Microbes and Infection,* entitled "The thymus as a target organ for mycobacterial infections". We show that the thymus is a target organ upon infection with *Mycobacterium tuberculosis, Mycobacterium avium* and *Mycobacterium bovis* bacillus Calmete-Guérin (BCG) and explore the kinetics of thymic infection with these three species of mycobacteria. The localization of the *bacilli* within the thymus is also addressed.

In chapter 3, we demonstrate that not *M. avium* only infected thymi maintain their ability to export new T cells but also show that T cells differentiated within infected thymi have an impaired ability to protect against *M. avium* infection. This work, "Dissemination of mycobacteria to the thymus renders newly differentiated T cells tolerant to the invading pathogen", will soon be submitted for publication. In this chapter, supplementary information regarding the role of newly differentiated T cells on the outcome of persistent *M. avium* infection is also presented.

In chapter 4 we explore the effect of antibiotic treatment on the control of *M. avium* growth in the thymus in comparison to the spleen, liver and lung. This work, entitled "Antibiotic Treatment has a Reduced Effect on *Mycobacterium avium* within the thymus", is being prepared for submission.

The general discussion of the work developed in this dissertation, as well as the final remarks, are presented in chapter 5.

CHAPTER 1

INTRODUCTION

1.1. MYCOBACTERIUM GENUS

The *Mycobacterium* genus comprises more than 120 species (1) from which, the great majority are non-pathogenic environmental mycobacteria. Some of the mycobacterial species are highly pathogenic such as *Mycobacterium tuberculosis, Mycobacterium leprae* and *Mycobacterium ulcerans*, the etiological agents of tuberculosis, leprosy and Buruli ulcers, respectively (2). Other species, such as *Mycobacterium avium* and *Mycobacterium intracellulare*, cause disease mainly in immunocompromised patients though they can also affect, to a much lower extent, healthy individuals (3).

Accordingly to the World Health Organization (WHO) it is estimated that about one third of the world population is infected with *M. tuberculosis*; 9,2 million new cases are diagnosed per year from which 8% arise in people infected with the human immunodeficiency virus (HIV). The acquired immunodeficiency syndrome (AIDS) has been highly associated with the increased prevalence of tuberculosis in developing countries (4) and also with the augmented number of people infected with *M. avium* in developed regions (Europe and United States) (3). Thus, *M. avium* and *M. tuberculosis* are certainly among the most relevant pathogens responsible for the morbidity and mortality of AIDS patients (5).

1.1.1. Mycobacterium avium - Epidemiology and pathogenesis

Mycobacteria such as *M. avium* and *M. intracellulare* are members of the *M. avium* complex (MAC), a group of environmental mycobacteria. These *bacilli* are extremely widespread in nature; they are common in surface waters and soils and are frequently isolated from water taps (2, 6, 7). Prevalence of MAC in municipal drinking water supplies is directly explained by their innate chlorine and biocide resistance (3).

Bacteria from MAC can cause disease in both animals and humans (6). They can be acquired from the environment either by inhalation of aerosols or by ingestion of contaminated water (8); animal-to-human or human-to-human transmission of this bacteria does not seem to occur (7, 8).

Infections caused by MAC in immunocompetent patients are mainly pulmonary (6, 9-11) though infection of soft tissue has been reported (7, 9). Several cases of mycobacterial lymphadenitis among children have also been described (12). Because bacteria from this complex are opportunistic pathogens, with the advent of AIDS epidemic, HIV infection has become the single most significant risk factor for MAC disease (6, 8, 9). In this scenario, disseminated infection is the most prevalent form of the disease (6, 8) specially in patients with profound immunodeficiency (<50 $CD4^+$ T cells per mm^3) (6).

The attenuated form of *Mycobacterium bovis*, the *M. bovis* bacillus Calmete-Guérin (BCG), is the only vaccine available to prevent mycobacterial infections. Although the efficacy of this vaccine varies widely in different regions of the world, and the protection conferred decreases significantly after the first 10 to 20 years of life, it is highly successful in preventing tuberculous meningitis and miliary tuberculosis in children (13). This vaccine does not seem to protect solely against *M. tuberculosis* infections as it also induces some cross-protection against MAC infections. One of the most relevant indications for this is the observed increased incidence of cervical lymphadenitis in children upon cessation of BCG vaccination in Northern Europe and in Czech Republic (14-16). Studies on the murine model have also shown that the rate of recovery of viable bacteria is lower in BCG-vaccinated mice than in non-vaccinated mice aerogenically challenged with *M. avium* (17, 18).

When a mycobacterial infection is established, treatment is quite challenging not only due to the intracellular localization of the *bacilli* within phagocytes (19) but also due to the biochemical characteristics of the bacteria, especially the cell wall (20), as it will be discussed over the next section. To overcome these difficulties, antibiotic combinations of 2 to 4 drugs, administered for long periods of time (6 to 12 months), are given in order to increase the microbicidal activity of the antibiotics that otherwise would only have a modest activity if used individually (19). Whereas for *M. tuberculosis* infections susceptibility testing using *in vitro* systems is extremely important in guiding treatment, for *M. avium* infections a predictable relationship between the results obtained in susceptibility testing *in vitro* and the clinical efficacy of the antibiotics is marginal (21). The *M. avium* infection can be, in most cases, treated with a combined therapy composed of a macrolide, such as clarithromycin or azythromycin, and ethambutol (to which a third or fourth drug are frequently added); nevertheless, recurrence and eventual development of resistance to antibiotics is not uncommon (7, 22). Aside from the low compliance to the long-term antibiotic treatment, serious toxicity side effects are also observed leading to the necessity of limiting the number of drugs, dose, and duration of therapy (7, 10).

1.1.2. The mycobacterial cell wall

One of the aspects of mycobacteria that is best studied is the structure and function of the cell wall, which confers to these bacteria their distinctive feature of acid fastness (ability to resist decoloration by alcohol-acid solution upon staining with carbolfuchsin) (6). The mycobacteria cell wall has exceptionally low permeability rendering mycobacteria

intrinsically resistant not only to common antibiotics but also to chemical disinfectants (20, 23, 24).

The mycobacteria cell wall is composed mainly by two segments (Fig. 1). The segment adjacent to the membrane is composed of peptidoglycan covalently attached to arabinogalactan which, in turn, is linked to mycolic acids (6, 25); this is termed the cell wall core – the mycolyl arabinogalactan-peptidoglycan complex (25). The other segment lays on the top of the cell wall core and is composed of lipids (with variable sized fatty acids) interspersed somehow with cell-wall proteins, phosphatidylinositol mannosides, phthiocerol-containing lipids, lipomannan and lipoarabinomannan (LAM) (25) (Fig. 1).

LAM is a component of the cell wall characteristic of the bacteria from the *Mycobacterium* genus. It is considered the most potent non-peptidic mycobacterial molecule able to modulate the immune response (26). In some species, such as *M. tuberculosis* and *M. avium,* LAM has a short oligosaccharide cap that allows it to bind to mannose receptors on macrophages enabling bacteria's phagocytosis by macrophages (25, 26).

Figure 1. Schematic representation of the mycobacterial cell wall. In the mycobacterial cell wall the peptidoglycan layer is linked to arabinogalactan which is then linked to high-molecular weight mycolic acids. This mycolyl arabinogalactan-peptidoglycan complex is covered with a layer of polypeptides and mycolic acids consisting of free lipids, glycolipids, and peptidoglycolipids. Other glycolipids include lipoarabinomannan and phosphatidyinositol mannosides (PIM). Porins are also present in mycobacterial cell wall and are required to transport small hydrophilic molecules through the membrane (scheme adapted from http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/u1fig11.html).

When the cell wall is disrupted (for instance, by extraction with various solvents), the free constituents are solubilised and the cell wall core is maintained. In a very simplistic way, in the mycobacterial cell wall, the soluble lipids, proteins and lipoglycans are the signalling, effector molecules in the disease process (25, 26), whereas the insoluble core is essential for cell viability of the bacteria and should be addressed in the context of new drug development (25). The unique components of the mycobacteria cell wall, as well as the enzymes involved on their biosynthesis, are excellent potential targets for highly selective and non-toxic drugs (6).

1.2. IMMUNE RESPONSE TO MYCOBACTERIAL INFECTIONS

Upon entering the organism, mycobacteria are phagocitosed by macrophages and dendritic cells (DCs). Afterwards, proteolytic fractions of the bacteria bound to the major histocompatibility complex (MHC) are presented at the cell surface of the phagocytic cell. This peptide-MHC complex (pMHC) forms the ligand that initiates the T cell response via interaction with the T cell receptor (TCR). In order to activate the T cell, the TCR has to be able to become activated upon recognition of both the host's MHC and of the bacterial derived-peptide, a situation termed MHC restriction (27).

DCs play a major role in the initiation of the acquired immune response to mycobacterial infections via interaction with $CD4^+$ T cells and subsequent activation of these cells. Consequently, $CD4^+$ T cells produce several cytokines that will be pivotal for the activation of bactericidal mechanisms of the infected macrophages. Over the next section, a more detailed overview of the players that participate in this immune response and the crosstalk between them will be made.

1.2.1. Recognition of the pathogen by phagocytes and activation of microbial killing mechanisms.

Mycobacteria are facultative intracellular pathogens. Although they infect mostly macrophages and DCs, other cell subsets might also be infected such as neutrophils (28-30), eosinophils (31), epithelial cells (32, 33) and adipocytes (34). Macrophages and DCs function simultaneously as host cells and as effector cells and play a key role in the initiation and direction of the innate and adaptive immune response (35).

Phagocytosis of mycobacteria by the host cells is an active process (35). It involves different receptors on the phagocytic cells including mannose receptors, the DC-specific intracellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN), surfactant

protein A, complement receptors (CR; CR1, CR3, CR4), mannose binding lectin and the class A scavenger receptor (35, 36). It has been shown *in vitro* that the use of multiple receptors by mycobacteria may lead to differences in signal transduction, immune activation and intracellular survival. However, the possible role of these events *in vivo* remains to be determined (2, 35).

Apart from phagocytosis, the recognition of mycobacterial components is of major importance in the establishment of an effective immune response. In this context, the different Toll-like receptors (TLR) recognise several mycobacterial constituents such as LAM, phosphatidylinositol mannoside (PIM), lipoproteins, heat-shock proteins and un-methylated CpG motifs in DNA (37-44). TLRs are expressed not only at the cell surface but also in phagosomes (35). After binding to TLRs, common signalling pathways lead to cell activation and production of cytokines such as interleukin (IL)-12 and tumour necrosis factor (TNF) (35).

Upon phagocytosis, the ingested material is contained within a phagosome that undergoes stepwise maturation. This process consists of progressive acidification of the compartment, by fusion with endosomes, and later with lysosomes, ultimately leading to the formation of the phagolysosome. Acidification of these structures is also achieved by the recruitment of vacuolar ATPase proton pumps. Phagolysosomes contain the enzymes and the required acidic environment to denature and degrade the engulfed material (45). However, virulent mycobacteria, such as virulent strains of *M. tuberculosis* and *M. avium,* have evolved survival strategies to interfere with the normal intracellular trafficking of vesicular compartments upon phagocytosis; they have the ability to inhibit the phagosome fusion with the lysosome preventing the formation of the phagolysosome (46-49) and the acidification of the vesicles (50). This inhibition is considered an active process controlled by the mycobacteria itself as only viable *bacilli* can accomplish it (46). However, *in vitro* activation of macrophages by the addition of interferon gamma (IFN-γ), TNF or granulocyte-macrophage colony stimulating factor (GM-CSF) can reverse the blockage of phagosome maturation (48). Interestingly, it was shown that when *M. avium*, but not *M. tuberculosis*, fails to prevent the phagosome-lysosome fusion its viability is not affected by the acidic environment of the mature phagolysosome (51).

Recognition of the mycobacteria by phagocytic cells leads to cell activation and production of cytokines, which in itself induces further activation and cytokine production in a complex process of regulation and cross-regulation (35). One of the first cytokines produced upon infection is TNF. This cytokine is produced by macrophages, DCs and T cells. Cells infected with mycobacteria produce TNF that has an autocrine activity in priming the cell's

microbicidal mechanisms together with IFN-γ. IFN-γ is mainly produced by $CD4^+$ T cells and NK cells though $CD8^+$ T cells and γδ T cells also produce it (52).

Both TNF and IFN-γ are essential for the control of mycobacterial infections (53). Mice deficient in the expression of one of these cytokines, or with their activity blocked, display increased susceptibility to *M. tuberculosis* (54-57) (Fig. 2), BCG (58, 59) or *M. avium* (60-62) challenge. In humans, gene mutations that lead to a deficient expression of the IFN-γ receptor have been shown to be associated with disseminated forms of mycobacteriosis (63, 64). In addition, anti-TNF therapy in patients with rheumatoid arthritis leads to an increased incidence of tuberculosis, probably due to the reactivation of latent *bacilli* (65).

Figure 2. Course of *M. tuberculosis* infection in the lung of WT and in gene-deleted mouse strains. To evaluate the role of different components of the immune systems on the susceptibility to *M. tuberculosis*, the course of infection was evaluated using a set of gene-disrupted mouse strains (aerosol inhalation of ~100 *M. tuberculosis* H37Rv *bacilli*/mouse). The growth rate of the pathogen has been described to be the same in all mouse strains until the third week of infection, when protective T-helper 1 (Th1) immunity begins to be expressed. In WT and T cell receptor (TCR) γδ KO mice *M. tuberculosis* infection is controlled and stabilizes after 3 weeks of progressive bacterial growth. Mice lacking the expression of β2-microglobulin (β2m) are also able to reach a bacterial plateau, although a few weeks later than WT mice. The worst progression of *M. tuberculosis* growth is observed for IFN-γ KO mice, followed by inducible nitric oxide synthase (iNOS) KO, TCRαβ KO, major histocompatibility complex (MHC) class II KO mice (MHC II KO). CFU: colony forming units. Figure adapted from North *et al.* 2004 (66).

TNF and IFN-γ exert their function, in part, by inducing the expression of inducible nitric oxide synthase (iNOS) (67, 68). iNOS is expressed exclusively upon activation of the macrophage and leads to the production of reactive nitrogen species (RNS) (69). Mice genetically modified that are unable to produce iNOS (iNOS KO) are more susceptible to *M. tuberculosis* infection; however, they are more resistant than IFN-γ KO mice (70) (Fig. 2). On the other hand, *M. avium* infected iNOS KO mice displayed unaltered (71) or even improved (72) clearance of the *bacilli* reinforcing that other IFN-γ-mediated mechanisms, yet to explore, underlie the ability to control bacterial growth in mycobacterial infections, especially by *M. avium*.

1.2.2. The pivotal role of T cells during mycobacterial infection

The emergence of HIV-mycobacteria co-infections has highlighted the role played by $CD4^+$ T cell during the effective immune response to *M. avium* and *M. tuberculosis* infections (73). In the murine model this has been proven by the increased susceptibility to *M. avium* and *M. tuberculosis* infections of mice depleted of $CD4^+$ cells (60, 74-76) and by the ability of $CD4^+$ T cells transferred from immunized mice to protect against *M. tuberculosis* challenge (77, 78).

Effective acquired cellular immunity to mycobacteria is critically dependent on the host's ability to initiate a T-helper 1 (Th1) cellular response (79). For an immune response mostly involved in the control of intracellular parasites, the priming of naive $CD4^+$ T cells requires that DCs, either infected or loaded with mycobacterial antigens, migrate from the site of infection to the draining lymph nodes (LN) (80-83) where they produce, among other cytokines, IL-12. This cytokine leads to the differentiation of $CD4^+$ T cells into a Th1 phenotype which means that they are mainly IL-2 and IFN-γ producers (84). Th1 cells are mostly involved in the control of intracellular parasites, as is the case of mycobacteria (85-87). This has been shown by Cooper *et al.* (85, 86) and Appelberg *et al.* (87, 88) for *M. tuberculosis* and *M. avium*, respectively. Both laboratories have observed that mice disrupted for the gene encoding the p40 subunit of IL-12 (IL-12p40 KO) are unable to control mycobacterial proliferation. Moreover, splenocytes from this mouse line were unresponsive to mycobacterial-specific antigens (86, 87). Additionally, it was shown that IL-12p40 KO mice were equally susceptible to *M. avium* infection as IFN-γ KO mice, indicating a crucial role of IL-12 in the development of a T cell population able to produce IFN-γ which mediates protection to mycobacterial infection. The phenotype observed for IL-12p40 KO mice could be reverted upon treatment of mice with recombinant IL-12 (87). The IL-12p40 subunit can be presented both as a homodimer [IL-12(p40)2] or as a

heterodimer [IL-12p70 (IL-12p35+IL-12p40)]. Moreover, IL-12p40 is also part of another cytokine, IL-23 (IL-12p40+IL-23p19). Interestingly, it has been shown that IL-12p40 KO mice have increased susceptibility to *M. tuberculosis* infection in comparison to IL-12p35 KO (89). In addition, in the absence of IL-12p35, IL-23 promotes IFN-γ production (90) which stresses the importance of the IL-12p40 subunit in the induction of IFN-γ production.

In the presence of IL-23, IL-6 and transforming growth factor beta (TGF-β), naive $CD4^+$ T cells differentiate into Th17, a T cell subset that produces mainly IL-17. It was shown that IFN-γ is able to regulate the induction of Th17 cells (91). In fact, during *M. tuberculosis* infection both IFN-γ and IL-17 producing $CD4^+$ T cells are expanded (90). Studies using mice infected with BCG, demonstrated that IFN-γ limits the differentiation of IL-17-producing T cell population and possibly, limits mycobacterial associated immune-mediated pathology (92). More recently, Khader *et al.* have shown that, although the immune response to *M. tuberculosis* infection is not significantly altered in the absence of Th17 cells, in vaccinated animals the absence of memory Th17 cells results in the loss of both the faster memory Th1 response and protection (93). They proposed that Th1 and Th17 responses cross-regulate each other during mycobacterial infection and this may have important immunopathologic consequences for the immune response against tuberculosis (77). Information is missing on the potential role of Th17 cells during *M. avium* infection.

Although the bulk of experimental data indicate that among T cells, the $CD4^+$ T lymphocytes play a major role in the immune protection against mycobacterial infection, other T cell subsets, such as $CD8^+$ T cells and γδ T cells, also participate in this process. $CD8^+$ T cells are activated by *M. tuberculosis* and by BCG infected cells through the presentation of mycobacterial antigens in the context of MHC class I molecules (94). Because $CD8^+$ T cells secrete IFN-γ and TNF in response to specific mycobacterial antigens (95) it has been suggested that these cells may contribute to protection by complementing $CD4^+$ T cells as a source of these cytokines (96). This hypothesis is supported by the work of Tascon *et al.* showing that the course of *M. tuberculosis* infection in H-2Aβ KO mice (mice that lack MHC class II expression) can be improved by transferring $CD8^+$ T cells from WT mice, but not from IFN-γ KO mice (97). Moreover, the protective effect of $CD8^+$ T cells can also be related with the cytotoxic characteristic of these cells as they express granzyme, Fas-L, granulolysin and perforin, which enable them to lyse infected macrophages (94) and induce protection *in vivo* (98). *In vivo* depletion of $CD8^+$ T cells during the course of *M. avium* infection has no impact on the bacterial load in the lung, liver or spleen (60, 99).

Data arising from humans clearly shows that γδ T cells participate in the immune response against mycobacteria, as evidenced by early accumulation of these cells in lesions and their *in vitro* expansion in response to mycobacterial non-peptide antigens (96). In humans, the majority of the circulating γδ T cells express the Vδ9 and the Vδ2 elements; *M. tuberculosis* readily activate $V\delta2^+$ T cells inducing IFN-γ production that can induce lysis of infected macrophages and help contain mycobacterial growth (100). In the murine model, the contribution of this cell subset is more controversial and seems to be dependent on the route of administration and number of viable *bacilli* delivered in the initial *inoculum* (101). In a single study performed by Ladel *et al.* it was shown that upon intravenous inoculation of a large *inoculum* of *M. tuberculosis,* TCR γδ KO mice rapidly succumb to infection but not when a lower dose was administered (101). In addition, TCRγδ KO mice are able to control *M. tuberculosis* infection upon challenge by aerosol (70) (Fig. 2). In the case of *M. avium* infection the role of γδ T cells is dependent on the virulence of the bacterial strain used: infection with a low-virulence strain induces an increased bacterial load in the lung of TCRγδ KO mice in comparison to WT, whereas this was not observed for a high-virulence strain of *M. avium.* Moreover, granulomas of TCRγδ KO mice infected with the high-virulence strain exhibit more compact lymphocytic lesions without extensive neutrophil influx or widespread tissue necrosis (102). The authors suggested that γδ T cells can be detrimental and appear to accelerate the destructive pathogenic effect of the response in infections with high-virulence strains of *M. avium* (102).

1.2.3. The establishment of persistent mycobacterial infections

Upon infection of WT mice, both with *M. tuberculosis* or *M. avium,* the bacterial load in infected WT animals rises clearly for the first weeks (103, 104). Afterwards, bacterial load stops rising or just slowly increases depending on bacterial and mouse strains and on the organs analysed. This change from fast to slow progression has been associated with the establishment of an acquired immune response, maintained mainly by $CD4^+$ T cells via IFN-γ production (60, 70).

The cellular response to *M. tuberculosis* and *M. avium* is relatively slow when compared to responses to viral or fast-growing bacterial infections (105). This may reflect an immunomodulatory activity of the pathogen or simply be a result of the dose and mode of administration of the bacteria or also be due to the slow division rate of the bacteria. In the case of a natural infection by *M. tuberculosis* very few *bacilli* are deposited in the alveolar space where the density of host macrophages and dendritic cells is low. These effects,

associated with the low rate of bacterial division, decreases the chances of rapidly having a great number of infected cells to promote a fast and strong stimulation of the immune system. Moreover, the adaptive immune response is only initiated in the draining LN upon dissemination of the *bacilli* (80) within dendritic cells (106). Recent work demonstrated that activation of $CD4^+$ T cells in the draining LN is the limiting step in the development of the adaptive immune response and, after the initial proliferation of $CD4^+$ T cells in the LN, effector cells traffic rapidly to the lungs and spleen (83, 107, 108). Results from different authors were unanimous in showing that *bacilli* do not reach the LN before day 10 of infection, following low dose aerosol infection, when the bacterial burden is already well established in the lung. By day 15 post infection, activated effector cells are found in the lung (107, 108) and around day 20 bacterial growth is arrested in this organ (107).

During the chronic phase of the infection, antigen-specific $CD4^+$ T cells are actively recruited and maintained in the lung. In this situation, $CD4^+$ T cells can be maintained either by proliferation (within the lungs or lymphoid organs) or could be continuously recruited from the naive recent thymic emigrant cell pool. However, 50 days after thymectomy, specific transgenic T cells were still present in the lung and no changes were observed in the bacterial load, suggesting that the thymic output of specific recent thymic emigrants is not relevant for the control of the infection during this period of time, at least for immunocompetent animals (109).

The establishment of persistent infections is thought to be, in part, dependent on the suppressive effect of regulatory T cells in an attempt to avoid exacerbated pathology (110). Initially, these cells were identified by the surface expression of CD25 however, it is now accepted that the hallmark of regulatory T cells is the forkhead box P3 (Foxp3) transcription factor (111). An increased proportion of this cell subset has been observed during experimental mycobacterial infections (112-114) and in patients infected with *M. tuberculosis* (115-118). Yet, among the few studies that have assess the role of regulatory T cells on the outcome of mycobacterial infections, most of them have relied on CD25 expression (113, 119); however, not all $CD4^+Foxp3^+$ regulatory T cells express CD25 (111). Recently, Scott-Browne *et al.* have used *M. tuberculosis*-infected mixed bone marrow chimeras in which $Foxp3^+$ cells were congenically marked enabling the depletion of this cell subset. In this system, they were able to show that depletion of regulatory T cells results in a modest reduction of the bacterial load (114) suggesting that regulatory T cells moderately affect the response of effector T cells in the context of *M. tuberculosis* infection in the murine model. *In vitro* studies suggest that this suppressive effect of regulatory T cells is also observed in *M. tuberculosis*-infected patients (116-118).

The ability of the bacteria to persist in the context of an apparently robust innate and adaptive immune response is also a matter of debate. Several hypothesis have been put forward such as: i) biochemical characteristics of the mycobacterial cell wall render bacteria highly resistant to anti-microbial compounds produced by the host cells; ii) bacteria can actively modulate antigen presentation (100, 120, 121) and the microbicidal function of the host cell (100, 122); iii) bacteria are able to enter into a quiescent, non-dividing state *in vivo* once their cell division is limited by the cellular immune response (123, 124) (it has been observed that 20 days post infection a decrease in the mRNA levels takes place for some of the most immunogenic antigens from *M. tuberculosis*) (123).

1.2.4. Containment of mycobacteria within granulomas

The protective immune response against most of the pathogenic mycobacterial infections relies in part on the formation of organized inflammatory lesions termed granulomas. For this reason, the immunological mediators involved in the induction of protective immunity are also major players in the immunopathology associated to mycobacterial infections; the lack of specific cell types or cytokines is responsible for altered granuloma assembly and/or maintenance (70, 125, 126).

The tuberculous granulomas are constituted by macrophages, T cells, B cells, DCs, neutrophils, fibroblasts and extracellular matrix components (2). The common model of a human tuberculous granuloma describes an area of central necrosis surrounded by a dense leukocyte wall that prevents mycobacterial spread (127). Some granulomas harbour mycobacterial material within their necrotic core though many of the necrotic areas are devoid of mycobacteria, as measured by immunohistological staining and by polymerase chain reaction (PCR) of microdissected material (127). Mycobacterial material was detected within APCs at the leukocyte infiltrates, indicating that antigen presentation takes place within specialized regions at the periphery of the classical granuloma structure (126, 128).

In humans, areas of caseous necrosis are typical not only during *M. tuberculosis* infections (128) but they can also be found in patients infected with MAC (129). In experimental models, these structures are formed upon *M. tuberculosis* infection of rabbits and guinea pigs (130, 131) and upon infection of mice with low doses of high-virulence strains of *M. avium* (126, 132). WT mice infected by aerosol with *M. tuberculosis* or WT mice infected with high doses of intermediate or high-virulence strains of *M. avium* do develop granulomas but with no caseous necrosis (130).

The granuloma has been considered pivotal in providing the framework whereby the acquired and the innate immune systems interact in order to create the required microenvironment for effective macrophage activation, prevent damage of the surrounding tissue and limit dissemination of the infection (127, 133). However, it has been proposed that this view of host-beneficial response to mycobacteria is too simplistic and that *M. tuberculosis* may also play a role in granuloma formation and maintenance ensuring disease progression (134). The role of the mycobacteria on the induction of granuloma formation is largely attributed to the cell wall lipids. It has been shown that these components, when inoculated within a gel matrix, are able to induce a proinflammatory response and leukocyte recruitment leading to the formation of structures in much similar to granulomas (135, 136)

The spatial localization of cells within the granuloma has been widely studied by standard histological and immunohistochemical methods (127, 137). However, these studies fail to provide information on cellular dynamics, in real-time, of these structures. Studies in transparent zebrafish embryo infected with *Mycobacterium marinum* have provided new insights on the early innate immune phase of macrophage migration and granuloma formation by showing the formation of this structure early before the onset of the adaptive immune response (138-140). Moreover, in this same model, Davis and Ramakrishnan showed that the recruitment of new macrophages to the nascent granuloma is highly dependent on the mycobacterial virulence locus ESX-1/RD1. These new macrophages phagocytose infected macrophages undergoing apoptosis, leading to the expansion of the number of infected macrophages and of bacterial numbers (141). By employing intravital multiphoton microscopy in the mouse model, Egen *et al.* have shown that in liver granulomas of BCG infected mice T cells are in constant motion throughout the lesion; each lymphocyte seems to wander through the entire granuloma likely making direct contact with most of the macrophages. Moreover, activated T cells enter the lesion but are restrained from leaving, accumulating within the granuloma (142). The authors propose that the retention of T cells is due to the macrophages acting as a scaffold upon which the lymphocytes crawl, rather than a physical barrier retarding lymphocytic egress (142). This study is pivotal in providing information on the dynamics of the adaptive immune response of the granuloma formation (143).

1.3. THE THYMUS AS AN ORGAN SUPPORTING T CELL DIFFERENTIATION

T cells are mainly generated within the thymus from bone marrow precursor cells that colonize the organ. Differentiation of thymocytes requires signalling between these cells and the different cell types encountered during differentiation. Medullary and cortical thymic epithelial cells (mTECs and cTECs, respectively) and thymic DCs are known to play a fundamental part in this process. Other cell types like macrophages, B cells and even re-circulating T cell are also known to influence T cell differentiation (144-147).

Thymocyte development starts with the entry of precursors from the bone marrow into the thymus, through post-capillary venules, at the cortico-medullary region. Upon arrival, they migrate to the sub-capsular zone through the cortex and then back, into the medulla, passing again through the cortex and the cortico-medullary regions (148-151) (Fig. 3). During this dynamic relocation, thymocytes undergo progressive developmental changes such that defined precursor stages appear in distinct areas of the thymus (148, 152). A few cell surface markers (CD4, CD8, CD3, CD25 and CD44) are used to define the main thymocyte populations (148). Upon entry into the thymus, precursor cells lack the expression of CD4 and CD8 and are termed double negative (DN) or $CD4^-CD8^-$ thymocytes (also referred as triple-negative thymocytes as they also lack the expression of CD3). The survival of DN thymocytes is supported by several cytokines. Among these cytokines, IL-7 is known to play a non-redundant role in T cell development; mice lacking IL-7 or IL-7-receptor have a marked reduction in $\alpha\beta$ T cells and lack $\gamma\delta$ T cells (153-156). Recently, it has been demonstrated that this cytokine is produced by TECs positioned at the cortico-medullary junction; these TECs bear markers both from mTECs and cTECs (157).

As DN thymocytes move to the sub-capsular zone, these cells undergo developmental programs through DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$) and DN3 ($CD44^-CD25^+$) during which V(D)J rearrangement at the TCR γ, δ and β loci occurs. Two lineages of T cell expressing either TCR$\alpha\beta$ or TCR$\gamma\delta$ diverge at the DN stage. The assembly of successfully rearranged TCRβ and the invariant pre-TCRα together with CD3 chains leads to the commitment to the TCR$\alpha\beta$ lineage and further differentiation to the DN4 stage ($CD44^-CD25^-$). At the DN4 stage, thymocytes express low levels of CD4, CD8 and CD3 and are immediate precursors of double-positive $CD4^+CD8^+$ (DP) thymocytes that migrate to the cortex (152).

Figure 3. Thymocyte migration and differentiation in adult thymus. Lymphoid precursors (LP) derived from the bone marrow enter the thymus trough the post-capillary venules and migrate outward as double negative (DN; $CD4^-CD8^-$) thymocytes toward the capsule in response to chemokine signalling. DN subsets are divided according to CD44 and CD25 the expression: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^-$) and DN4 ($CD44^-CD25^-$). At the DN3 stage, rearrangement of the TCRβ chain takes place; at this point, commitment into naturally killer (NK) T cells, γδ T cells or αβ T cells takes place. Successful rearrangement of the TRCβ chain with the pre-Tα chain directs thymocytes to the DN4 stage where a high-rate proliferation is observed. In the cortex, double positive (DP; $CD4^+CD8^+$) thymocytes migrate randomly before positive selection and then reduce their mobility as they undergo positive selection mediated by cortical epithelial cells (cTECs). At this point, thymocytes whose TCR fail to interact with peptide-MHC complexes expressed by cTEC die due to the lack of stimuli (death by neglect). Following positive selection, thymocytes migrate to the medulla and, once there, single positive (SP; $CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes are screened for reactivity to self-antigens, including tissue-specific antigens (TSAs), expressed by medullary epithelial cells (cTECs) and cross-presented by DCs. Potential autoreactive thymocytes are eliminated, mainly by clonal deletion. Finally, fully mature T cells exit the thymus via blood and/or lymphatic vessels.

After progression to the DP stage and rearrangement of the TCRα locus, subsequent developmental decisions of thymocytes are determined by the interaction of the TCR with the pMHC complexes (158, 159). At this stage, thymocytes go through one of three possible fates: death by neglect, positive selection or negative selection. Failure to express a TCR or the expression of a TCR that is unable to interact with pMHC ligands, results in death by neglect due to the lack of survival signals. The cell being positively *versus* negatively selected relies essentially on the strength of the interaction of the TCR with the pMHC complexes (159, 160). DP thymocytes expressing TCRs that bind pMHC complexes with low affinity/avidity are rescued through positive selection and progress with differentiation into self-MHC-restricted thymocytes. Negative selection eliminates thymocytes that express TCRs that establish within the thymus high-affinity/avidity interactions with pMHC complexes reducing the chances of full differentiation for auto-reactive T cells. Altogether, these processes generate a repertoire of T cells that is, for the vast majority, self-restricted and self-tolerant (160).

The main mechanism of negative selection is through clonal deletion. However, receptor editing (enhancement of secondary TCR rearrangement and replacement of an autoreative TCR) and anergy (state to non-responsiveness to antigen) are also possible mechanism to get rid of auto-reactive T cells (144, 161).

What is the signal that drives the cell through the decision of positive *versus* negative selection is a matter of debate. Some authors proposed that the strength of the TCR-pMHC complex interaction is based on the number of TCRs occupied: low doses of high affinity peptide (occupying few TCRs) should lead to positive selection (162, 163). However, this view has been questioned by others; it has been proposed that an individual receptor could distinguish ligand affinity by “reading” its occupancy time – kinetic proofreading. Because of the shortest occupancy time, low-affinity ligands would be limited to induce only early TCR signals, leading to positive selection, whereas high-affinity ligands contact with the TCR for a longer time and induce late TCR signals that would initiate negative selection (160, 164-166).

Compartmentalization of the selection processes has been a highly controversial field. However, the notion that clonal deletion occurs mainly at the thymic medulla prevails for several reasons. First, the expression of the TCR is not fully upregulated on DP thymocytes in the cortex. For this reason, a bias towards low-avidity interactions with pMHC ligands on cortical epithelial cells may favour survival (positive selection) rather than death (negative selection) of the thymocytes. Second, it is only upon the transition from the cortex to the medulla that thymocytes fully up-regulate the TCR. Moreover, it is only in the medulla that DCs and mTECs have their co-stimulatory molecules fully up-

regulated which is also necessary for the interaction. This, again, provides a bias towards high-avidity signals in the medulla. Third, the medullary microenvironment provides a unique representation of the self-antigens (167). Nonetheless, evidence exists showing that negative selection can efficiently occur in the thymic cortex via cortical DCs (168-170).

The promiscuous expression of proteins characteristic of peripheral organs is the hallmark of mTECs. This expression enables mTECs to present a large proportion of the tissue-specific antigens (TSAs), in the context of MHC molecules, to the developing thymocytes. This results ultimately in the elimination of a great proportion of the potentially auto-reactive thymocytes and generation of a T cell repertoire that is, to a vast extent, tolerant to self (167). Only one molecular determinant was found, so far, to regulate the expression of TSAs in mTECs - the autoimmune regulator, AIRE (171). Mutations in the gene encoding AIRE underlie a specific human autoimmune disease, the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (172, 173). Later studies have shown that clonal deletion of TSAs-specific T cells was impaired by AIRE deficiency (174-176).

In addition to mTECs, thymic DCs also participate in the establishment of self-tolerance in thymic medulla (177-179). Gallegos *et al.* have shown that thymic DCs can pick TSAs from mTECs, process and cross-present them to developing thymocytes (177). The rational for this discovery was based in two main observations: i) the frequency of mTECs that express a given TSA is low (from 1 to 5%); ii) TECs are poor at presenting antigens to induce tolerance (180). Acquisition of TSAs by DCs from mTECs can be either from apoptotic mTECs or from viable cells via secreted exosomes or through gap junctions (167). Moreover, it has also been shown that DCs loaded in the periphery with antigens can also be recruited to the thymic medulla; this contributed to the negative selection of thymocytes with specificities for antigens transported by the DCs and that are not expressed within the thymus (178, 181-183)

Differentiation of naturally occurring regulatory T cells within the thymus is also dependent on the TCR-pMHC complex interactions (111, 184) and requires the up-regulation of the transcription factor Foxp3 to commit to the regulatory T cell lineage (185). It has been proposed that regulatory T cells are selected by high-affinity agonist self-ligands (144, 186-188). The timing and location of suppressor lineage commitment within the thymic microenvironment is still under discussion (167). Due to the requirement of antigen-specific stimulation of the TCR it is tempting to assume that mTECs mediate regulatory T cell lineage commitment in order to generate self-specific regulatory T cells. Nevertheless,

evidence exists supporting the contribution of mTECs (111, 189-191) cTECs (191-195) and/or DCs to this process (191, 196, 197).

1.3.1. Thymic output throughout life

Early in life, thymic export establishes the size and the diversity of the human naive T-cell pool. However, upon puberty, the thymus undergoes a physiological involution and its activity decreases. For this reason, many have questioned the relevance of this organ and the contribution of the newly generated T cells during adulthood.

One of the classical ways of assessing thymic function is by the quantification of TCR excision circles (TRECs). TRECs are episomal DNA by-products excised during the rearrangement of the gene segments encoding the TCR (198, 199). Because TRECs are stable, do not duplicate during mitosis and are diluted with each cellular division, they have been proposed as a marker to evaluate recent thymic emigrants in the periphery and active ongoing thymopoiesis in the thymus (200). However, this technique has several caveats: i) the number of TRECs depends not only on thymic output, but also on the longevity of naive T cells that contains TRECs (200, 201); ii) increased expansion of T cells can induce changes in TREC proportion; iii) quantification of TRECs requires cell disruption to assess DNA content disabling the simultaneous identification of recent thymic emigrants and measurement of their function (200). The recently described CD31 (202) and the protein tyrosine kinase 7 (PTK7) (203), the only cell surface markers available for human $CD4^+$ T cell recent thymic emigrants identified until now, will be extremely helpful to overcome these difficulties. No cell surface markers were identified to date for mouse recent thymic emigrants. However, intrathymic injection of fluorescent dyes (such as CSFE or FITC) (204), labelling of thymocytes with BrdU (205) and, more recently, the use of transgenic mice that carry the green fluorescent protein (GFP) driven by the recombination-activation gene 2 (RAG2) promoter (RAG2-GFP) have enabled the labelling of recent thymic emigrants and their functional study (206, 207).

In humans, thymic involution is characterized by a progressive replacement of the perivascular space with adipocytes. Despite this replacement, the remaining cortical and medullary tissue in the aging thymus is histologically normal (208-210). In seeking the relevance of this remnant tissue, it was shown that new lymphocytes continue to emigrate from the thymus during adulthood though at lower numbers than the observed in pre-pubescent children (211-213). However, despite the decreased number of TRECs per gram of thymic tissue, the proportion of TRECs per 10^5 human thymocytes is maintained constant until, at least, the fifth decade of the human life (208).

The question remains of whether the impairment on thymic output is merely quantitative or is it also qualitative. It was shown that T cell precursors from old mice, when transferred to young mice, generate defective $CD4^+$ T cells (evaluated by a decreased calcium influx upon TCR cross-linking, decreased IL-2 production and proliferation); the same was observed when T cell precursors from young mice were transferred to old mice (214). This observation reinforces previous observations that precursors from old mice are defective (215, 216) and also that the thymic environment from old mice does not provide the adequate signals for normal differentiation. Previous reports showed a decrease in expression of various epithelial cell genes, that include IL-7, keratin-8 and Foxn1 occurs with age, which may be related to the impaired thymic function (157, 217, 218).

1.3.3. The role of the thymus in T cell reconstitution upon lymphopenia

To facilitate cardiac transplantation, performed due to congenital heart disease or cardiomyopathy, infants under the age of one year are partially or totally thymectomyzed. Later in life, this intervention has been shown to cause modest defects in the T cell compartment (slight decrease on the number of T cells) and little or no impact on the well-being of the individuals (no increased number of infection episodes were observed in thymectomyzed children up to 10 years after thymectomy) (219-223). These studies indicate that, even though the thymus retains its ability to actively produce T cells during adulthood, the T cell repertoire of humans is already formed at birth, and that T cells proliferate as needed to compensate for the lack of thymic output in these situations (223).

In situations of severe T cell depletion (due, for example, to HIV infection, cytoreductive transplantation or chemotherapy regimens) the role of the thymus in T cell homeostasis seems to be critical and the degree of insufficiency of the adult thymus is apparent (224-227). Studies on T cell reconstitution after lymphopenia demonstrate that the greater abundance of thymic tissue is associated with improved recovery of total and naive $CD4^+$ T cells (228-230), increase frequency of TRECs (231, 232) and increased TCR repertoire diversity (224, 231). For all this, several strategies have been explored to enhance T cell production by the thymus in adults such as administration of IL-7, of growth hormone and sex steroid modulation (233, 234). Still, immune reconstitution may not always be possible especially in some cases of HIV^+ patients. This could be due to age or to viral injury of the thymus, as will be discussed in the next section. In these situations, thymic transplantation has been attempted both in patients undergoing (235) or not highly active antiretroviral therapy (HAART) (236-238). Thymic transplantation has yielded promising results in patients with DiGeorge syndrome (congenital disorder that affects the heart, parathyroid

glands, and thymus; in complete DiGeorge syndrome, patients have severely reduced T-cell function) (239-242) however, in HIV$^+$ patients rejection of the thymic grafts was observed and, in patients that were not on HAART, viral-infection of the thymic graft occurred (235-238).

1.4. INFECTION OF THE THYMUS

1.4.1. Infection of the thymus: human case reports

Studies on the immunology of infectious diseases focus mainly on the effector immune response in peripheral lymphoid organs and at the sites of infection, as well as on vaccine development. However, studies on the infection of the thymus itself and potential effects of such infection are scarce, though this scenario is progressively changing.

Only a few case reports have described infection of the thymus by mycobacteria in humans (Table 1) (243-251). Specimens were usually obtained from surgical interventions due to suspicion of a thymoma upon computerized tomography (CT) scan. However, posterior histological analysis of the resected material revealed the presence of foci of caseation and multiple granulomas with extensive central necrosis in the thymus. The presence of these structures was a constant in all the reports published of thymic mycobacterial infection in humans. However, not all the reports have confirm the presence of the *bacilli* either by positive cultures of thymic tissue, detection of acid fast *bacilli* by Ziehl-Neelsen staining or by PCR (Table 1).

Unlike infection of the thymus by mycobacteria in humans, HIV-1 infection of the thymus has been widely demonstrated. This was observed in specimens obtained *post-mortem* or upon cardiac surgery (252-258). Early HIV infection of the thymus was associated to increased lymphoid infiltrate of the thymic perivascular space in comparison to age-matched controls. In late HIV-infection, the changes on thymic morphology resembles, but is more exaggerate than, the normal atrophy associated with age. In addition to thymic atrophy, HIV-infected thymi exhibit islands of thymic epithelium devoid of lymphocytes that are surrounded by a dense lymphoid infiltrate of CD8$^+$ cytotoxic T cells, monocytes and B cells (226, 256, 259). Of notice, even in patients with profound lymphopenia in late stages of AIDS, small islands with active thymopoiesis were retained within the thymus (226).

Studies in humanized mice [severe combined immunodeficiency (SCID) mice transplanted with human foetal liver and thymus under the kidney capsule: SCID-hu mice] (260-262) and in human foetal thymic organ cultures (FTOCs) (263, 264) have also confirmed the presence of HIV in the thymus and the associated thymic atrophy. Thymi from non-human

primates infected with simian immunodeficiency virus (SIV) were also positive for the presence of the virus (265-267).

Over the next section, an overview on the several experimental models of thymic infection as well as the potential impact of such infections will be presented.

Table 1. Described human case reports of thymic infection by mycobacteria. All the reports had in common the presence of foci of caseation and multiple granulomas with extensive central necrosis in the thymus.

Reference	*AFB*	*Culture*	*PCR*	*Observations*
Duprez *et al.*, 1962 (243)	-	-	n.a.	
Silvola and Lahdesmaki, 1966 (244)	-	-	n.a.	PPD positive.
FitzGerald *et al.*, 1992 (245)	+	+	n.a.	HIV-seronegative patient. Sputum smears after thymectomy were negative for AFB but grew *M. tuberculosis.*
Simmers *et al.*, 1997 (246)	+	-	-	PPD positive.
Stephen *et al.*, 2003 (247)	-	+	n.a.	Sputum smear negative for AFB.
Kpodonu *et al.* 2005 (248)	-	n.a.	-	Asymptomatic for tuberculosis. Negative for PPD. No contact with patients with tuberculosis.
Sacco *et al.*, 2004 (249)	-	-	n.d.	Primary pulmonary tuberculosis. Poor response of the thymic lesions to anti-mycobacterial therapy
Ganesan and Ganesan, 2008 (250)	+	+	n.a.	HIV-seronegative patient. Good response to anti-mycobacterial therapy.
Prabhu *et al.*, 2008 (251)	n.a.	n.a.	n.a.	Completely treated pulmonary tuberculosis in childhood.

AFB: acid fast *bacilli*; PCR: polymerase chain reaction; PPD: purified protein derivative test; n.d.: not detected; n.a.: not assessed; +: positive; -: negative.

1.4.2. Animal models of thymic infection – consequences of having a pathogen within the thymus

Several reports demonstrate that the thymus is a target organ upon experimental infection by a quite diverse set of microorganisms including viruses, parasites, bacteria and fungi (Table 2). A common feature of most of these models of thymic infection is the premature thymic atrophy reflected in the reduced thymic weight and cellularity. In cases where histological analysis was performed, atrophic thymi exhibit a clear depletion of cortical thymocytes as well as the loss of the cortico-medullary boundary. This atrophy was, for most cases, related to increased apoptosis of the DP thymocytes (Table 2). Interestingly, some reports showed premature thymic atrophy with one strain of a certain pathogen (in

the case of *Francisella Tularensis,* SIV or measles virus infections) whereas, when a different strain was used, infection of the thymus was still observed but with no premature thymic atrophy (265, 268, 269). Moreover, in the case of *Trypanosoma cruzi* infections, several factors could lead to a reversion of the premature thymic atrophy such as parasitaemia decrease (270), administration of benznidazole (a drug used for the treatment of *T. cruzi* infections) (271) or blockage of *trans*-sialidase (an enzyme secreted by the parasite) (272). Altogether, these reports indicate that factors derived from the microorganism may be responsible, although not exclusively, for the observed thymic atrophy.

Host factors may also be involved in premature thymic atrophy induced by the infection. Some reports present evidence that corticosteroids are related to infection-induced atrophy (268, 273). Infectious diseases are, in general, regarded as promoting stress and, consequently, inducing an increase of serum corticosteroid levels (274) that is responsible for augmented apoptosis of DP thymocytes (275, 276). In infections by *T. cruzi* and *F. tularensis* an increase in serum corticosteroid levels was observed; inhibition of corticosteroid signalling (induced by adrenalectomy plus blockage of corticosteroid receptors) reverted thymic atrophy (268, 273). Another death-pathway that may be involved in thymocyte apoptosis is via signalling through the TNF. Thymic atrophy in *F. tularensis* infection was not observed in mice deficient for TNF-receptors 1 and 2 indicating that, besides corticosteroids, TNF is also mediating thymic atrophy in this model (268, 277). This pathway was not involved in *T. cruzi* infection (273). Thus, several mechanisms, host or pathogen derived, may underlie premature thymic infection upon infection. These mechanisms are not completely elucidated and may vary according to the infecting agent.

Table 2. Reports describing premature thymic atrophy upon infection of the thymus.

Infectious agent		Model	Cortical atrophy(1)	DP depletion	Reference
Protozoa	*Trypanosoma cruzi*	Mouse	+ (2)	+	(278-280)
	Plasmodium berghei	Mouse	+	+	(281)
Bacteria	*Francisella tularensis*	Mouse	+/-(3)	+/-(3)	(268)
Fungi	*Paracoccidioides brasiliensis*	Mouse	+	n.a.	(282)
Virus	Murine Leukemia virus (MLV)	Mouse	+	+	(283, 284)
	Measles virus	SCID-hu mouse	+/-(3)	+/-(3)	(269)
	Simian Immunodeficiency Virus (SIV)	Macaques	+/-(3)	+/-(3)	(265)
	Human Immunodeficiency Virus (HIV)	SCID-hu mouse	+	+	(260-262, 285)

n.a.: not assessed; DP: $CD4^+CD8^+$ thymocytes; SCID: Severe combined immunodeficiency; (1) Assessed histologically; (2) Reverted upon clearance of the pathogen; (3) Depends on the strain of the infectious agent.

Other outcomes of thymic infection, in addition to premature thymic atrophy, have been reported. Upon *T. cruzi* infection, conventional histology revealed that the parasite was detected in clusters in the thymic parenchyma; *in vitro* studies revealed that both DCs and TECs could become infected (278). Changes in the TEC phenotype and/or distribution were observed with the infection: surface markers specific for cTECs were observed in the medulla and, simultaneously, markers of mTECs were detected in the thymic cortex (278). Moreover, when the expression of extracellular matrix ligands in the thymus was assessed, Cotta-de-Almeida *et al.* have observed a progressive increase in the deposit of fibronectin and laminin, and respective receptors, during the course of infection, reaching a maximum at the peak of parasitaemia and thymic atrophy (280). Mendes-da-Cruz *et al.* also observed an increase in chemokine (CXCL12 and CCL4) deposition as well as an up-regulation of the receptors (CXCR4 and CCR5) upon *T. cruzi* infection (286). These observations reported by Cotta-de-Almeida *et al.* and by Mendes-da-Cruz *et al.* paralleled an increased intrathymic thymocyte migration ability (280, 286) as well as an increased frequency of immature DP T cells in the peripheral lymphoid organs in infected animals (286). It was also shown that in the LN of infected mice, these immature DP cells, as well as some mature $CD4^+$ T cells, exhibited the Vβ5 TCRβ-chain, a segment that is not detected in the SP populations of the infected thymi. The authors suggest that DP thymocytes abnormally released from *T. cruzi* infected thymus have evaded negative selection thus bearing a potential autoreactive phenotype that, apparently, differentiate in the periphery into $CD4^+$ T cells (287). Supporting these observations, $CD4^+$ T cell mediated autoreactivity against myocardial cells has been experimentally demonstrated during *T. cruzi* infection (288, 289).

1.5. AIMS

The main goal of this thesis is to explore the impact of the infection of the thymus by mycobacteria on the immune response to the infection. Specifically, we proposed to:

I. Characterize the kinetics of thymic infection upon challenge with different mycobacterial species; localize and identify the infected cells within the thymus;
II. Assess the potential impact of the mycobacteria within the thymus on T cell differentiation and on the ability of newly differentiated T cells to mount an immune response to mycobacterial infections.
III. Study antibiotic efficacy on bacteria within the thymus in comparison to other organs.

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CHAPTER 2

THE THYMUS AS A TARGET ORGAN FOR MYCOBACTERIAL INFECTIONS

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The thymus as a target for mycobacterial infections

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Abstract

Mycobacterial infections are among the major health threats worldwide. Ability to fight these infections depends on the host's immune response, particularly on macrophages and T lymphocytes produced by the thymus. Using the mouse as a model, and two different routes of infection (aerogenic or intravenous), we show that the thymus is consistently colonized by *Mycobacterium tuberculosis*, *Mycobacterium avium* or *Mycobacterium bovis* BCG. When compared to organs such as the liver and spleen, the bacterial load reaches a plateau at later time-points after infection. Moreover, in contrast with organs such as the spleen and the lung no granuloma were found in the thymus of mice infected with *M. tuberculosis* or *M. avium.* Since T cell differentiation depends, to a large extent, on the antigens encountered within the thymus, infection of this organ might alter the host's immune response to infection. Therefore, from now on, the thymus should be considered in studies addressing the immune response to mycobacterial infection.
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Keywords: *Mycobacterium avium*; *Mycobacterium tuberculosis*; BCG; Thymus; T lymphocytes

1. Introduction

Humans all over the world are frequently exposed to bacteria from the *Mycobacterium* genus. This genus includes several pathogens that represent a public health issue worldwide. *Mycobacterium tuberculosis*, the pathogen responsible for tuberculosis in humans, is the leading cause of death from a curable infectious disease [1,2]. About two decades ago tuberculosis gained a renewed significance in Europe and in North America. This resulted mainly from the raise in disease incidence due to the spread of HIV infection [1]. Despite being generally viewed as a lung disorder, *M. tuberculosis* can affect any organ. The latter assertion is particularly relevant for AIDS patients, for which tuberculosis is frequently extrapulmonary [2]. $CD4^+$ T lymphocytes depletion in AIDS patients is clearly associated with increased susceptibility to infection with *M. tuberculosis* as well as with *Mycobacterium avium*, a bacterium that otherwise rarely causes a serious threat to humans with a healthy immune system [3]. In fact, disseminated infections with *M. avium* are common in advanced immune-deficient HIV-infected patients that do not receive anti-mycobacterial therapy, as it is still the case in most developing countries [4].

Both *M. avium* and *M. tuberculosis* are facultative intracellular pathogens. These bacteria invade important cells of the immune system: macrophages and dendritic cells. The immune response to fight these pathogens depends mainly on

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the ability of macrophages to destroy intracellular bacteria. Various cells of the immune system have been shown to play a role in this process [5]. Among these, T lymphocytes, especially $CD4^+$ T cells, are essential players. T cells generated in the thymus have the ability to recognize infected macrophages and stimulate their mycobacteria killing properties. Although the function of $CD4^+$ T cells to control mycobacterial infections has been recognized for a long time, the association of AIDS with mycobacterial infections strengthens, nowadays, the importance of these cells [2].

The only vaccine in use to prevent mycobacterial infections is the *M. bovis* bacillus Calmette–Guérin (BCG). BCG is a live attenuated form of *M. bovis* used to prevent tuberculosis. Protection conferred by BCG depends mostly on the ability of the bacteria to establish a pool of protective memory mycobacterial-specific T lymphocytes. Although the efficacy of this vaccine varies greatly in different regions of the world, in those areas in which it is effective, infants are protected from tuberculous meningitis and miliary tuberculosis, the most dangerous forms of the disease [6,7]. However, protection is known to drop with age and BCG does not protect adults from tuberculosis. The mechanism underlying the loss of BCG-induced protection in adults is a matter of intense debate and strategies to generate a more protective vaccine are, presently, among the most relevant health research topics [6].

Several reports in the last few years showed that the ability of the thymus to produce new T cells after antiretroviral therapy in AIDS patients is extremely important for the reconstitution of the host's immune system [8]. In human, as well as in mouse models of infection, mycobacteria disseminate from the original site of infection to several other organs, including organs of the immune system like lymph nodes and spleen. As the central organs where new T cells are generated and where differentiating T cells learn how to distinguish self- from non-self-antigens, infection of the thymus could perturb T cells differentiation and the production of new naïve T cells. Although thymic infection has been rarely reported in humans, but given the potential implications to the host's immune response, we decided to investigate how frequently mycobacteria disseminate to the thymus in mouse models of mycobacterial infection.

The present study shows that the thymus is a target for mycobacterial infection by the three most relevant mycobacteria – *M. tuberculosis*, *M. avium* and BCG. The importance of this finding stands from the fact that the ability to fight mycobacterial infections depends to a large extent on T cells, and that the repertoire of T cells is strongly influenced by the antigens they encounter in the thymus during differentiation.

2. Materials and methods

2.1. Mice

Mice used were 8–10-week-old female mice from the C57BL/6, BALB/c, 129/Sv or DBA2 strains (purchased to Harlan, Barcelona, Spain); and from the immune-deficient mouse line $TCR\alpha^{-/-}$ [9] on a C57BL/6 background (purchased from Jackson Laboratory, Bar Harbor, ME). All mice were maintained in a specific-pathogen-free animal house and experiments were conducted in accordance with National and European Union guidelines for the care and handling of laboratory animals. Mice, under 12-h light cycle, were given sterile chow and tap water ad libitum. Four to ten animals were used per experimental group for each time-point.

2.2. Experimental infection

M. avium strain 2447 (smooth transparent variant kindly provided by Dr. F. Portaels from the Institute of Tropical Medicine, Antwerp, Belgium), *M. avium* strain 25291 (smooth transparent variant obtained from the American Type Culture Collection – ATCC, Manassas, VA) or BCG (strain Pasteur 1172P2) were grown in Middlebrook 7H9 medium containing 0.05% Tween 80 at 37 °C until mid-log phase of growth. Bacteria were harvested by centrifugation and resuspended in saline containing 0.04% Tween 80. The bacterial suspensions were briefly sonicated with a Branson sonifier to disrupt bacterial clumps, diluted, and stored in aliquots at −70 °C until use. Intravenous infection with *M. avium* or with BCG was performed through the tail lateral vein with 10^6 colony forming units (CFU) per animal.

M. tuberculosis strain H37Rv (obtained from the Pasteur Institute, Paris, France) was grown in Proskauer Beck medium containing 0.01% Tween 80 at 37 °C to mid-log phase. Bacteria were harvested by centrifugation, resuspended in sterile water, and stored in aliquots at −70 °C until use. Aerogenic infection was performed using an airborne infection apparatus (Glas-col Inc., Terre Haute, IN, USA). The nebuliser compartment was filled with a suspension of *M. tuberculosis* at a concentration calculated to result in the implantation of 20 bacilli, on average, in the lung of each animal, as previously described [10].

At specific time-points, the bacterial load in the organs was determined as previously described [10,11]. Briefly, mice were anesthetized with isoflurane and retro-orbital bleeding was performed before sacrifice, also with isoflurane. The organs were removed in aseptic conditions, homogenized, and serial dilutions were prepared in distilled sterile water with 0.05% Tween 80 and plated onto Middlebrook 7H10 agar medium for *M. avium*, or in Middlebrook 7H11 agar medium for *M. tuberculosis* and BCG. 7H10 plates were incubated for 1 week whereas 7H11 were incubated for 3 weeks at 37 °C and the numbers of CFU counted.

2.3. Histology

Thymi were removed from C57BL/6 mice at different time-points post-infection, fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Five micrometre sections were stained with the Ziehl–Neelsen method using standard procedures.

2.4. Gene expression analysis

Thymi and spleens were collected from non-infected mice and from mice intravenously infected for 4 weeks with *M. avium* and frozen at −70 °C until use. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into first-strand DNA using the superscript first-strand synthesis system for reverse-transcription polymerase chain reaction (PCR) (Invitrogen) according to manufacturer's instructions. The expression level of the *IFN-γ* gene and of the reference gene hypoxanthine guanine phosphoribosyl transferase (*HPRT*) were studied by real-time PCR using SYBER green (Qiagen, California, USA) in a LightCycler Instrument (Roche, Indianopolis, USA). Specific oligonucleotides were used for *IFN-γ* (sense: 5′-TGG CAA AAG GAT GGT GAC ATG-3′; anti-sense: 5′-GAC TCC TTT TCC GCT TCC TGA-3′) and for *HPRT* (sense: 5′-GCT GGT GAA AAG GAC CTC T-3′; anti-sense: 5′-CAC AGG ACT AGA ACA CCT GC-3′) genes. The cycling parameters, for both target genes, were one cycle of 95 °C for 15 min, 40 cycles of 94 °C for 15 s, 58 °C for 20 s and 72 °C for 15 s, followed by one cycle of 65 °C 1.5 min and 35 °C 1.5 min.

3. Results

3.1. The thymus is a target organ for M. avium

When C57BL/6 mice were intravenously infected with a medium virulence *M. avium* strain (2447), the number of bacteria reached a plateau at about 4 weeks post-infection in the liver and spleen but continued to rise for several more weeks both in the lung and in the thymus (Fig. 1A). At late time-points post-infection these two organs presented a very similar bacterial load. When organ weights were taken into account, bacterial loads per gram of infected tissue were much higher in the thymus than in the lung at late time-points (lung: 198 ± 36 mg; thymus: 40 ± 8 mg). One day after intravenous inoculation, bacteria were found in the lung, liver and spleen of all animals, while no bacteria were detected in the thymus of 22% of the mice (Fig. 1B). Even in thymi that were infected at this early time-point, the bacteria load was

Fig. 1. The thymus is a target organ for *M. avium*. C57BL/6 mice were intravenously infected with 10^6 CFU of *M. avium* strain 2447. (A) Bacterial load was determined at different time-points (1 day, 4, 9, 12, 17, 42 and 52 weeks) in spleen, liver, lung and thymus. Each point represents the average ± SD log10 CFU from 6 to 10 mice. (B) The number of CFU in the thymus was determined 1-day post-infection in 37 mice. Each bar represents the number (and percentage) of animals presenting a defined numbers of CFUs.

much lower than in the other organs studied. At 4 weeks after infection bacterial colonization of the thymus became clear in all animals. Progressive thymic colonization by *M. avium* was not associated with a high bacterial load in the blood as virtually no bacteria were detected in the blood both at day 1 and at 4 weeks after infection (from 150 ml of blood analyzed: 1 CFU was detected in 5 out of 16 mice 1 day post-infection; and up to 3 CFU in 4 out of 15 mice 4 weeks post-infection; no bacteria were detected for the other 22 mice). The ability of *M. avium* to colonize the thymus was independent of the host genetic background since a similar course of infection was observed for different mouse strains (129/Sv, BALB/c and C.D2) (data not shown). The thymus was also a target organ for infection when a more virulent strain of *M. avium* (25291) was used (data not shown).

To better understand the lack of control of the bacterial load at 4 weeks post-infection in the thymus when compared to organs like the spleen, we evaluated the *IFN-γ* gene expression in both organs. At this time-point we detected a marked up-regulation in the *IFN-γ* gene expression in the spleen of infected mice, as measured by real-time PCR in relation to the reference gene HPRT (infected: 17.02 ± 9.45; non-infected: 0.78 ± 0.29). In the thymus, corroborating the lack of bacterial load stabilization at this time-point, we detected a weaker up-regulation of the *IFN-γ* gene expression (infected: 0.23 ± 0.11; non-infected: 0.05 ± 0.02).

3.2. The thymus is colonized by M. tuberculosis upon aerogenic infection

Intravenous infection of mice with *M. avium* is frequently used to study mycobacterial disseminated chronic infections [12]. However, this route of bacterial inoculation does not represent a natural route of infection. In order to understand whether the thymus is also a target organ for mycobacteria when a natural route of infection is used, we studied mice aerogenically infected with *M. tuberculosis*. As can be observed in Fig. 2, infection of the thymus became well established in all animals studied 3 weeks after infection with *M. tuberculosis*. Mycobacterial growth was arrested in the lung 3 weeks after aerogenic infection while, in the thymus, it progressed for at least 9 weeks after infection (Fig. 2). The ability of *M. tuberculosis* to colonize the thymus was also observed for different mouse strains (DBA/2 and 129/Sv) (data not shown). Whereas in *M. avium*-infected mice the bacterial load in the thymus reached almost 10^6 CFU and stabilized at 16–18 weeks after infection, in *M. tuberculosis*-infected animals, the bacterial load stabilized when the organ contained 10^3–10^4 CFU.

3.3. Mycobacteria preferentially infect the thymic medulla

Thymic sections, obtained at different time-points post-infection, were stained using the Ziehl–Neelsen method, to determine the localization of *M. avium* within the thymus. Eight weeks post-infection the microscopic structure of the thymus did not differ from the controls (Fig. 3A). Only a small number of infected cells were detected (Fig. 3B,C), which is in accordance with the low bacterial load of the organ at this time-point. These few infected cells were mostly seen at the cortico-medullary junction and each cell contained only a few bacteria (Fig. 3B,C). At 24 and 28 weeks post-infection aggregates of infected macrophages with large cytoplasm were observed essentially in the medulla and in the cortico-medullary regions (Fig. 3D,G). These aggregates became more evident at later time-points (Fig. 3G), and the number of bacilli per cell appeared also to increase over time (Fig. 3E,F,H,I). It is important to note that even at these late time-points it is still possible to clearly distinguish the cortex and the medulla, even though the overall structure of the thymus became modified due to the presence of a larger number of macrophages. Even considering that granulomas are difficult to observe in lymphoid organs, it is interesting to note that a layer of lymphocytes

Fig. 2. The thymus is a target organ for *M. tuberculosis*. C57BL/6 mice were aerogenically infected with *M. tuberculosis* in order to have on average 20 CFU per lung at day 1 post-infection. Bacterial load was determined at 1 day, 3, 9, 18 and 22 weeks post-infection in lung, spleen and thymus. Each point represents the average ± SD log10 CFU from four mice per group from one out of two independent experiments. Dashed lines represent the detection limit.

Fig. 3. Representative Ziehl–Neelsen staining of thymi from mice intravenously infected with *M. avium*. (A, B, C) Eight weeks post-infection the thymus architecture is well preserved with cortex (c) and medulla (m) being very well defined (A); bacteria were essentially found at the cortico-medullary junction, and each infected macrophage contained only one or a few bacilli (arrows) (B, C). (D, E, F) Twenty-four weeks post-infection the thymus presented aggregates of macrophages that were present essentially at the cortico-medullary region and in the medulla (aggregates of macrophages are indicated by *); higher magnifications of these thymi show that the macrophages in the small (E) and large (F) aggregates were for the most part infected. (G, H, I) Twenty-eight weeks post-infection the thymi contain an increased number of macrophage aggregates (indicated by *); higher magnifications of these small (H) and large (I) aggregates of macrophages show that most of the cells were infected with a great number of bacilli. (J, K) Aggregates of infected macrophages were frequently found in the vicinity of a blood vessel. (L) High magnification of a blood vessel of a thymus from an immune-deficient mouse (TCR$\alpha^{-/-}$) 24 weeks post-infection showing infected macrophages in close proximity to a blood vessel and even in a sub-endothelial position. c: Cortex; m: medulla; bv: blood vessel; and * aggregates of macrophages.

was not observed surrounding the aggregates of macrophages. This was particularly evident for infected macrophages present in the medulla of the thymus.

Careful analysis of several slides from different time-points post-infection (4, 8, 12, 24 and 28 weeks) failed to detect extracellular bacteria or bacteria in the cytoplasm of cells other than macrophages. We paid particular attention to blood vessels and to the surrounding tissue. As can be observed in Fig. 3J,K aggregates of macrophages were often detected in the proximity of but never in the lumen of the blood vessel, neither bacteria were detected inside endothelial cells. To better address this issue we analyzed the thymus of immune-deficient mice (TCR$\alpha^{-/-}$ mice do not have functional T cells due to an inserted mutation in the constant region of the T cell

receptor [9]) in which bacterial load is higher than in wild-type infected animals. In these mice the infected cells are easier to detect due to the increased bacterial load, which make them a valuable model to carefully analyse the blood vessels and surrounding tissues. As can be observed in Fig. 3L infected cells were frequently found in the vicinity of blood vessel and even in the sub-endothelial region. Again, bacteria were never seen in the lumen of blood vessel or inside endothelial cells.

During aerosol infection with *M. tuberculosis* the bacterial load in the thymus stabilized at lower bacterial numbers when compared with the intravenous infection with *M. avium*. Consequently, bacteria were more difficult to detect inside this organ, even at late time-points post-infection. Even though the structure of the organ was well preserved at all time-points post-infection (Fig. 4A), a small number of macrophage aggregates were observed in the medulla and in the cortex (in some cases from the cortico-medullary region to the sub-capsular zone) (Fig. 4A). *M. tuberculosis*-infected macrophages were detected mostly scattered or forming small aggregates in the medulla (Fig. 4B,C), but also in the cortex (Fig. 4D) and in the sub-capsular zone (Fig. 4E,F).

3.4. The bacterial load in the thymus of BCG-infected mice reaches a plateau at later time-points than in other organs

Knowing that both the pathogenic *M. tuberculosis* and the opportunistic *M. avium* do colonize the thymus and that the increase in bacterial numbers is halted at later time-points in this organ, we next investigated whether non-pathogenic BCG also has the ability to colonize the thymus. As previously described by others, the BCG load started to decrease 2–3 weeks after inoculation in the spleen, liver and lung [13]. In contrast, bacterial load in the thymus continued to increase for at least 9 weeks after infection (Fig. 5).

4. Discussion

In this report we show that the thymus, the central organ where T cells learn how to distinguish self- from non-self-antigens, is colonized by the three most relevant mycobacterial species – *M. tuberculosis*, *M. avium* and BCG. Moreover, within the thymus, bacterial load is still increasing at times when this has stabilized in other organs; particularly striking is the case of BCG. Indeed, BCG starts to colonize the thymus by the time bacterial load is already decreasing in other organs. This finding might have important implications on the host's immune response to infection by mycobacteria.

The thymus has been frequently studied during infection by HIV and other viruses, both in human and animal models [8,14]. This organ has also been studied as a target organ in animal models of infection by *Trypanosoma cruzi* and *Paracoccidioides brasiliensis* [15]. However, mycobacterial infection of the thymus has only been reported in the 1960s in experiments that used extremely high bacterial doses (lethal within a few weeks) and that, for this reason, cannot be

Fig. 4. Representative Ziehl–Neelsen staining of thymi from mice aerogenically infected with *M. tuberculosis* for 22 weeks. (A) Low magnification at late time-points post-infection shows that the architecture of the thymus was, for the most part, well preserved and that small aggregates of macrophages were present in the medulla and in the cortex. (B, C) Higher magnifications show that macrophages infected with *M. tuberculosis* in the medulla contain essentially only one bacilli per cell (B) or a small number of bacteria per cell (C). (D) Infected cells were also found scattered in the cortex. (E, F) Aggregates of macrophages were present in the cortex, from the cortico-medullary region to the sub-capsular zone (F); higher magnification showing one infected macrophage inside an aggregate of macrophages in the sub-capsular region. c: Cortex; m: medulla; and * aggregates of macrophages.

Fig. 5. Increase in BCG CFU is halted in the thymus at later time-points than in other organs. C57BL/6 mice were infected intravenously with 10^6 CFU of *M. bovis* BCG. The bacterial load was determined at 1 day, 2, 4, 8 and 12 weeks post-infection in spleen, liver, lung and thymus. Each point represents the average ± SD log10 CFU from six mice per group from one out of two independent experiments. * Indicates no viable bacteria were detected in the thymi at 1 day and 2 weeks post-infection.

considered adequate to study the natural course of infection [16,17]. In humans, the presence of mycobacteria in the thymus has been described only in a few case reports [18–20]. We now show in mice, that mycobacteria reach the thymus of all animals infected aerogenically with *M. tuberculosis* or intravenously with BCG or *M. avium.* It is therefore possible that infection of the thymus in humans has been underestimated. Since mycobacteria can disseminate in humans from the original site of infection to other organs, that might also be the case for the thymus. It is therefore relevant to clarify how frequently the thymus is involved during mycobacterial infections/dissemination in humans. Since, in mice, infected thymi do not show signs of granuloma, imaging studies in humans might not be conclusive to address this question. Post-mortem analysis of human thymi, either by mycobacterial culture or by tissue staining might otherwise be more informative, particularly in tuberculosis endemic areas.

Another aspect that still needs to be clarified relates to how bacteria travel from the original site of infection to other organs including the thymus. The thymus has for long been seen as an immune privileged organ, partially isolated from the bloodstream due to the presence of the blood-thymus barrier. Even so, since the first evidence for its existence, the blood-thymus barrier was shown to be mostly confined to the thymic cortex [21]. Moreover, it is also known that the thymus is continuously colonized by cells from the bone marrow (mostly early T cell precursors and monocytes/macrophages) that enter the thymus mainly through the cortico-medullary region [22]. Therefore, we hypothesise two different sources of mycobacteria disseminating into the thymus: (a) free bacteria circulating in the bloodstream and (b) bacteria circulating within monocytes/macrophages. Since we observed that almost 80% of the mice intravenously infected with *M. avium* are colonized one-day post-infection, it is reasonable to envisage that bacteria can enter the thymus directly crossing the blood vessels. In support of this route of access there are recent reports showing that mycobacteria can infect endothelial cells [23]. If so, this must occur at a very low frequency since careful analysis of the thymus and other organs, at various time-points post-infection, did not reveal any bacteria in the lumen of the blood vessels or inside endothelial cells. In fact, 1-day post-infection, most of the infected thymi presented only one to three bacteria (Fig. 1B). Therefore, this might not be the only route for thymic infection. In accordance, at 8 weeks post-infection with *M. avium*, the infected cells perceived in the thymus were at the cortico-medullary junction, the region of the thymus where cells are known to enter the organ [22]. In addition, in thymi from immune-deficient mice, we found heavily infected cells in the vicinity of blood vessels and even in sub-endothelial regions; further suggesting that circulating infected cells can reach the thymus. Altogether, the evidence supports that not only single bacteria infect and proliferate inside macrophages upon crossing the blood vessels, but also that already infected monocytes/macrophages directly enter the thymus.

As we show here, both pathogenic and non-pathogenic mycobacteria can reach and reside within the thymus. In addition, the rise in bacterial load within the thymus is generally arrested at late time-points during infection when compared with other target tissues. These observations were more significant for BCG infection. Mice are known to control the infection with BCG in organs such as the lung, spleen and liver [13]. However, our data show that the bacterial load in the thymus is still rising 2 months after intravenous infection, while in organs such as spleen and liver the bacterial load started to decrease as early as 2 weeks post-infection. Also, in mice infected with *M. avium*, the bacterial load stops rising in the spleen and liver as soon as 4 weeks post-infection, whereas in the thymus, the number of bacteria in the organ stabilises several weeks later. The reduced up-regulation of INF-γ in the thymus when compared to the spleen supports the late control of the bacterial load in the thymus. This difference might

be due to differences on the T cells or on the antigen-presenting cells. In fact, macrophages and dendritic cells have, in the thymus, a quite different function when compared to organs such as the lymph nodes or the spleen. Within the thymus, these cells essentially present self-antigens providing signs for differentiation (positive or negative selection signals) to the differentiating thymocytes. This observation may be explained by mycobacteria which remain undetected and therefore escape effective immune responses for longer periods.

Whether infection of the thymus relates with the protection conferred by BCG against *M. tuberculosis* remains to be elucidated. Since intravenous administration of BCG is commonly used to study protection in mouse models of tuberculosis, it will be interesting to investigate whether thymic colonization by BCG influences the generation of protective T cells.

It should therefore be considered whether mycobacteria inside the thymus influence T cell selection during differentiation. Immature thymocytes are known to go through a process of positive and negative selection. Thymic selection aims at generating a pool of useful T cells: T cells whose T cells receptors are able to recognize and become activated when interacting with self major histocompatibility complex molecules (MHC) expressing foreign peptides (self-restricted), but that are tolerant to self-MHC molecules expressing self-peptides (self-tolerant) [24,25]. Thymocytes expressing a newly rearranged TCR reside inside the thymus for a period estimated to last from 2 to 3 weeks before being exported to the peripheral lymphoid organs [26]. TCRs with high affinity for MHC-peptide complexes encountered in the thymus are known to be eliminated through negative selection [24,25]. Negative selection occurs mainly in the medulla and cortico-medullary regions, areas of the thymus where we found most of the bacteria. The interaction of differentiating thymocytes expressing a T cell receptor that recognizes infected cells can certainly have immunological consequences. If this is the case, the interaction promotes either positive or negative selection of mycobacteria-specific T cells. Considering the distribution of bacteria we found within the thymus, it is most likely that negative selection takes place, which should next be investigated. Importantly, an increasing body of data in humans and in mice shows that despite the physiologic thymic atrophy that occurs normally during ageing, new T cells can be produced and play an important role in the immune response to infection throughout life [27–30].

In light of the present findings, the thymus should from now on be thoroughly investigated as a target for mycobacterial infection. Furthermore, the repertoire of T cells generated by an infected thymus and its influence on the course of a chronic infection or on a process of tuberculosis re-infection or reactivation deserves attention.

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CHAPTER 3

MYCOBACTERIA WITHIN THE THYMUS IS RESPONSIBLE FOR THE SHAPING OF THE PERIPHERAL IMMUNE RESPONSE

3.1.

DISSEMINATION OF MYCOBACTERIA TO THE THYMUS RENDERS NEWLY GENERATED T CELLS TOLERANT TO THE INVADING PATHOGEN

Dissemination of mycobacteria to the thymus renders newly generated T cells tolerant to the invading pathogen

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Running foot: Thymic infection with mycobacteria

ABSTRACT

The immune response to mycobacterial infections relies mostly on the activation of infected cells, macrophages and dendritic cells, by $CD4^+$ T lymphocytes. The ability of the thymus to generate a population of T cells that is, for the vast majority, self-restricted and self-tolerant depends on the antigens encountered during differentiation. We have recently shown that mycobacteria disseminate to the thymus, which raised the question on how mycobacteria within the thymus influence T cell differentiation. By transplanting *Mycobacterium avium* infected thymi under the kidney capsule of nude mice, we now show that newly generated T cells are unable to mount a protective immune response against this pathogen. These *de novo* generated T cells seem otherwise normal, since they retain the ability to reconstitute a peripheral T cell population, to respond to unspecific stimuli *in vitro* and to specific stimuli like OVA upon *in vivo* immunization. The observation that infection of the thymus interferes with T cell differentiation, generating T cells that are tolerant to pathogen-specific antigens, is of relevance to understand the immune response during chronic persistent infections. In addition, it has potential implications for the repertoire of T cells generated in patients recovering from severe lymphopenia, such as patients infected with human immunodeficiency virus with a mycobacterial co-infection receiving anti-retroviral therapy.

INTRODUCTION

Several mycobacterial species are important human pathogens, causing a diverse set of diseases that are usually chronic and progressive (1). The host immune response against these infections depends to a great extent on the activation of infected cells, mostly macrophages and DCs, by mycobacteria-specific $CD4^+$ T cells. IFN-γ produced by mycobacteria-specific T cells plays a central role in this activation and, consequently, in the control of mycobacterial growth (2, 3). The raise of *Mycobacterium avium* and *Mycobacterium tuberculosis* infections in HIV^+ patients with low $CD4^+$ T cell counts strengthened the relevance of this cell population (4). Unlike *M. tuberculosis*, a primary human pathogen, *M. avium* is a common opportunistic bacteria responsible for localized or disseminated infections in immune-suppressed individuals (1).

In addition to causing pathology at the site of entry (lung, gut, skin) mycobacteria are able to disseminate to several other tissues or organs such as pleura, meninges and, to a lesser extent, bones and joints (1). A few recent case reports have shown the thymus as an additional site for mycobacterial dissemination (5-9). Using the mouse as a model, we showed previously that the thymus is a target for mycobacterial dissemination, regardless of the route of infection being intravenous or aerogenic (10). In both cases, the bacterial load is initially undetectable or extremely low and it rises progressively for several weeks post infection (10).

Because infection of the thymus with several pathogens is accompanied by premature thymic atrophy, associated or not with progressive loss of peripheral T cells, research on the topic has focused essentially on this aspect (11). However, previous studies have shown that although disseminated infection with a highly-virulent strain of *M. avium* is accompanied by an intense premature thymic atrophy, this seems not to be the case when a less virulent strain of *M. avium* is used (12). The possibility that T cell differentiation is maintained regardless of the progressive colonization of the thymus with a given pathogen prompted us to investigate to which extent T cell differentiation is preserved and whether the newly generated T cells, whose differentiation occurs within infected thymi, differ from those generated in non-infected thymi. We observed that, although the production of new T lymphocytes is maintained by infected thymi, these T cells do not mount a protective immune response against the same pathogen in peripheral organs.

MATERIAL AND METHODS

Mice

57BL/6 (WT), nude (B6.Cg-*Foxn1*^{nu}/J) and TCR$\alpha^{-/-}$ (B6.129S2-Tcratm1Mom/J) mice were purchased from Charles River Lab, Taconic and The Jackson Lab, respectively. All animal experiments were performed in accordance with National and European Union guidelines for the care and handling of laboratory animals, and have been approved by the National Veterinary Directorate and local Animal Ethic Committee.

Infection and determination of bacterial load

Female mice, 8-10 weeks old, were i.v. infected with 10^6 CFU of *M. avium* strain 2447 (provided by Dr Portaels). Mice were sacrificed with isoflurane. The bacterial load in the organs was determined as previously described (10).

Thymic transplant

Thymi were aseptically removed from TCR$\alpha^{-/-}$ mice and maintained in DMEM (supplemented with 10% heat inactivated FCS, 10mM HEPES, 1mM sodium-pyruvate, 2mM L-glutamine, 50μg/ml streptomycin and 50U/ml penicillin), for 10-20min until being transplanted under the kidney capsule of nude mice (anaesthetized i.v. with 200μg xylazine hydrochloride and 100mg ketamine hydrochloride).

Immunization

Mice were immunized 3 times, at 1 week intervals, subcutaneously on the back with 10µg OVA (Sigma-Aldrich) emulsified in 250µg dimethyldioctadecyl ammonium chloride (Sigma-Aldrich) and 25µg monophosphoryl lipid A (Sigma-Aldrich), prepared as specified elsewhere **(28)**. Mice were sacrificed 1 week after the last immunization.

Immunofluorescence

histopathology and image analysis. Sections (7μm) of cryopreserved thymi were fixed in cold acetone, washed and blocked with 4% BSA in PBS 0.05% Tween. Tissues were incubated overnight at 4ºC with purified antibodies [anti-mycobacteria spp, anti-CD11c (N418) and anti-F4/80 (A3-1) (Serotec) or anti-EpCam (G8.8, BD Biosciences)]. Secondary antibodies used were: anti-rat IgG Alexa Fluor 594; anti-rabbit IgG Alexa Fluor

488 (Molecular Probes); biotinilated anti-hamster IgG (Serotec) plus Pacific Blue-conjugated streptavidin (Molecular Probes). iNOS (clone M-19, Santa Cruz Biotechnology) detection was performed in paraffin embedded liver sections as described elsewhere (29). Slides were visualized using an epifluorescence microscope (BX61 microscope combined with an Olympus DP70 camera) and images were analysed using Image J software. No significant signal was observed in the negative controls (no antibodies, no primary antibodies and isotype controls).

Morphometric analysis was performed in liver sections stained with haematoxylin-eosin. Using the 20x objective, 5 random fields (in a total area of 2.9mm^2) were photographed from one liver section per mouse. Quantification of the liver areas occupied with inflammatory lesions was performed using the Cell^P software. Classification of the lesions was performed blindly.

Cell preparation, *in vitro* stimulation and ELISPOT

Cell suspensions were prepared by gentle disruption of the organs between two notched slide glasses. Erythrocytes were lysed using a hemolytic solution (155mM NH_4Cl, 10mM $KHCO_3$, pH 7.2) and cells were resuspended in supplemented DMEM.

Spleen cells were stimulated *in vitro* as described elsewhere (22). Stimuli used were Con A (4μg/ml), *M. avium* total extract proteins (4μg/ml) or a panel of molecular weight fractions from *M. avium* short term culture filtrate (2μg/ml) (details on the preparation as supplementary data, Fig. S2) (22). IFN-γ was quantified by ELISA (R4-6A2 as capture and biotinilated AN18 as detection antibodies; BD Biosciences). Assay sensitivity was 20pg/ml.

ELISPOT was performed as described previously (29). The stimuli used were either 10μg/ml of the mycobacteria epitope Ag85A$_{241-260}$ (Metabion) (30) or OVA (Sigma-Aldrich).

Flow cytometry

Cells were labeled with antibodies specific for CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7, BioLegend), CD62L (Mel14, eBiosciences) and CD44 (IM7, BD Biosciences). Cell acquisition was performed on a FACscalibur flow cytometer or on a FACSAria cell sorter (Becton Dickinson) using Cell Quest software. Data were analyzed using FlowJo software.

Statistical analysis

Differences between means of experimental groups were analyzed with two-tailed Student's t-test. Differences with a $P \leq 0.05$ were considered significant. Significance was referred as * when $P \leq 0.05$, as ** when $P \leq 0.005$ and as *** when $P \leq 0.001$. Results and

DISCUSSION

Infected cells within the thymus are CD11c$^+$

To evaluate the potential impact of mycobacterial dissemination to the thymus on T cell differentiation, the identification of the infected cells, as well as their localization, is essential. The thymus becomes progressively colonized (Fig. 1A), from almost undetectable bacterial loads on day 1, to approximately 100 bacilli at 4 weeks, and roughly 10^5 viable bacteria 22 weeks post i.v infection with 10^6 CFU of *M. avium*. As infection progress, bacteria are detected mostly in clumps of large cells at the cortico-medullary region and within the medulla (Fig. 1B). All infected cells stain for CD11c (Fig. 1C, D, E) and consist of two populations. The large majority are CD11c$^+$F4/80$^+$ (Fig. 1C), a phenotype consistent with foamy macrophages (13, 14). To a lesser extent bacteria were detected within CD11c$^+$F4/80$^-$ cells (Fig. 1D), a phenotype compatible with DCs. No infected EpCam$^+$ cells were observed (Fig. 1E) suggesting that thymic epithelial cells (TECs) are not a target for *M. avium* infection.

DCs within the thymus play an important part in central tolerance processes (15-17). Moreover, adoptively transferred DCs, loaded with antigens, have been shown to migrate to the thymus and participate in tolerance induction (15, 16). Recently it was shown that DCs of extrathymic origin are mainly localized at the cortico-medullary region (18). The observation that, in infected mice, *M. avium* containing-DCs are present in the cortico-medullary region and in the medulla, suggest that thymic infection might have an impact on T cell selection.

T cells generated in mycobacteria-infected thymi are unable to control the growth of this pathogen in the periphery

Accelerated thymic atrophy, which is accompanied by reduced or abrogated T cell differentiation, has been frequently observed during systemic infections (11). This is well known in humans infected with HIV (19) and has been shown in several animal models of infection (11, 12). The mycobacterial infection used in this work does not result in altered total thymic cell number up to 22 weeks post infection (Fig. S1A). Only minor differences were detected, at late time points, on the proportion of the 4 main thymocyte populations (CD4$^-$CD8$^-$, CD4$^+$CD8$^+$, CD4$^+$CD8$^-$, CD4$^-$CD8$^+$) (Fig. S1B). Moreover, no differences were detected in the amount of T-cell receptor rearrangement excision circles (TRECs) up to 30 weeks post infection (Fig. S1C). These results suggest that thymocyte differentiation is maintained throughout chronic mycobacterial infection, despite the presence of a considerable number of mycobacteria-infected cells within the thymus.

We next asked to what extent newly generated T cells, whose differentiation occurred within infected thymi, are able to mount a protective immune response against mycobacterial infection. To characterize T cells whose differentiation occurred within infected thymi, and their ability to control mycobacterial growth, it was necessary to create a system in which these cells could be analyzed with no interference from T cells whose differentiation occurred prior to thymic infection. To do so, a thymic lobes from *M. avium* infected TCR$\alpha^{-/-}$ mice, was transplanted under the kidney capsule of nude mice. Nude mice have no thymus but have competent T cell precursors (20) and TCR$\alpha^{-/-}$ mice have a thymus but their T cell precursors are unable to fully differentiate (21). By performing the thymic lobes transplantation we generated mice with T cells derived from nude's bone marrow that differentiated exclusively within *M. avium* infected TCR$\alpha^{-/-}$ thymi. Nude mice transplanted with non-infected thymi were used as controls (Fig. 2A). To guaranty a similar bacterial load in the peripheral organs of nude mice transplanted with infected or non-infected thymi, at the moment of peripheral T cell colonization, all animals were infected 3 days post transplantation (Fig. 2A). At 5 weeks post transplantation, similar numbers of newly formed $CD4^+CD3^+$ and $CD8^+CD3^+$ T cells were detected in the blood of mice transplanted with infected or non-infected thymi (Fig. 2B). At sacrifice (7 to 9 weeks post infection, depending on the experiment), thymic engraftments under the kidney capsule were confirmed macroscopically and microscopically for each animal. The numbers of T cells in the spleen (Fig. 2C), as well as the activation profile of the $CD4^+$ T cells (Fig. 2D) was similar for mice transplanted with infected or non-infected thymi. Thus, T cells arising from infected or non-infected thymi are able to colonize the periphery to the same extent. However, when the bacterial load was assessed, mice transplanted with non-infected thymi presented almost 10-times less viable bacteria in the liver and spleen than those transplanted with infected thymi (Fig. 2E). Interestingly, the bacterial load in the liver of mice transplanted with infected thymi was not different from that in nude mice. These observations clearly indicate that T cells differentiated within infected thymi have an impaired ability to protect against the invading pathogen in peripheral organs.

T cells that differentiate in an infected thymus have a reduced ability to produce IFN-γ in response to *M. avium* antigens

The impaired ability of T cells arising from infected thymi to mount a protective immune response against *M. avium* might be due to a general defect of these cells to respond to stimuli or to a specific *M. avium* induced tolerance. As the immune response to mycobacterial infections depends greatly on the ability of mycobacterial-specific T cells to

produce IFN-γ (2, 22), splenocytes were stimulated *in vitro* (Fig. 3A) with a panel of *M. avium* antigenic fractions (Fig. S2). Splenocytes from WT as well as from nude mice transplanted with non-infected thymi produced similar amounts of IFN-γ upon stimulation with Con A and *M. avium* antigenic fractions (Fig. 3A). In contrast, splenocytes from mice transplanted with infected thymi generated undetectable levels of IFN-γ for most of the antigenic fractions tested. As expected, splenocytes from non-transplanted nude mice did not produce detectable levels of IFN-γ (unpublished data). Altogether, these results shown that T cells generated within infected thymi are specifically unable to respond to *M. avium* antigens.

In our thymic transplant system, an innate immune response to infection is taking place when de novo generated T cells colonize the periphery. Even though our results suggest that thymic export from infected and non-infected thymi is similar (as shown by no premature thymic atrophy; no differences on the amounts of TRECs in the thymus; and similar peripheral T cell reconstitution in mice transplanted with infected or non-infected thymi) we cannot exclude the possibility that a difference in the timing of thymic export exists which, if so, could delay the acquired immune response in one of the experimental groups. To exclude this possibility, purified $CD4^+$ T cells from mice transplanted for 9 weeks, were transferred to new $TCR\alpha^{-/-}$ mice (Fig. 3B). These mice were infected 3 days after the adoptive transfer and sacrificed 18 weeks later. T cells transferred from both types of mice reconstituted equally well $TCR\alpha^{-/-}$ mice (Fig. S3). When stimulated *in vitro* with *M. avium* antigens, splenocytes from mice that received $CD4^+$ T cells from nude mice transplanted with infected thymi produced extremely low levels of IFN-γ, when compared to mice that received $CD4^+$ T cells from animals transplanted with non-infected thymi (Fig. 3C). Further supporting the idea that T cells differentiating in infected thymi are specifically impaired in their ability to respond to mycobacterial antigens was the observation that mice transplanted with infected thymi mount an immune response to OVA indistinguishable from that of mice with non-infected thymi (Fig. 4).

Differentiation within infected thymi generates T cells unable to participate in the formation of granulomas

The histological analysis of the inflammatory lesions offers significant insight on the participation of antigen-specific T cells in the immune response to mycobacterial infection. While antigen-specific T cells are required for the formation of fully mature granulomas, in T cell-deficient hosts only poorly-organized granulomas are observed, provided that there is IFN-γ secretion by innate immune cells (23). The liver inflammatory lesions of nude

mice transplanted with infected or non-infected thymi, as well as the non-transplanted nude and WT mice, were classified taking into consideration their organization as: inflammatory infiltrates (lymphocytes and/or macrophage-like cells showing no signs of organization), poorly-organized granulomas (with a central core of macrophage-like cells surrounded by lymphocytes, but not forming a clear cuff) and well-organized granulomas (a central core of macrophage-like cells surrounded by a well-defined cuff of lymphocytes) (Fig. 5A, right, middle and left panels, respectively). While the 3 types of lesions are present in the liver of WT infected animals, well-organized granulomas, which depend on the existence of antigen-specific T cells, are virtually absent from nude mice, as described previously (23). Mice transplanted with infected thymi present the same pattern of lesions as nude animals (Fig. 5B). These observations corroborate the finding that T cells arising from infected thymi are mostly depleted of active mycobacteria-specific T cells. The inability of T cells from mice transplanted with infected thymi to participate in the formation of structured granulomas is further supported by the lower number of liver lesions expressing inducible nitric oxide synthase (iNOS), an enzyme whose expression is up-regulated in activated macrophages (12), when compared to WT or to mice transplanted with non-infected thymi (Fig. 5C and D).

The similarities in the ability of infected and non-infected thymi (or of the isolated T cells recovered from transplanted recipients) to repopulate the periphery, as well as their indistinguishable ability to produce IFN-γ in response to Con A, strongly argue against a general anergy of T cells arising from infected thymi. Moreover, the observation that T cells that differentiate in infected thymi mount an immune response to OVA similar to that of T cells that differentiate in non-infected thymi (Fig. 4) strongly suggests that the differentiation within infected thymi gives rise to T cells that are specifically impaired in their ability to respond to *M. avium* antigens. Whether this tolerance to mycobacterial antigens is due to clonal deletion, anergy of *M. avium*-specific T cells and/or the generation of a specific regulatory T cell population is not clear (24). The observation that mice transplanted with infected or non-infected thymi present similar numbers of regulatory T cells ($CD3^+CD4^+Foxp3^+$) suggest that the difference does not stand on the proportion of regulatory T cell produced (unpublished data). Although we cannot exclude the possibility that differentiation within infected thymi generates a population enriched in mycobacterial-specific regulatory T cells, our histological analysis does not support that. Regulatory T cells are normally found in granulomas induced by *M. tuberculosis* (25). In our experiments the pattern of lesions in mice that received infected thymi was very similar to that of nude mice, which suggests the absence of specific T cells, whether regulatory or conventional. Clonal deletion of specific T cells within the thymus is more

likely to be the mechanism responsible for the observed tolerance leading to their reduced recruitment or residence in the granulomas. In favor of this hypothesis is also the observation that bacteria within the thymus are found mainly in the medulla and cortico-medullary regions where negative selection mostly occurs.

Although infection of the thymus has previously attracted the curiosity of researchers, the work we present here is, to our knowledge, the first demonstration that bacteria within the thymus disturb T cell differentiation and influences their ability to mount an effective immune response against the infecting pathogen. T cell tolerance to an invading microorganism might be detrimental, especially in situations in which the peripheral T cell population depends to a great extent on thymic export, as we show in our animal model. In humans, despite the progressive physiologic thymic atrophy with age, it has become clear that the thymus maintain its ability to generate new T cells in adulthood. This is of great relevance in situations of T cell reconstitution upon severe lymphopenia such as in patients with AIDS, submitted to cytoreductive transplant, or to chemotherapy (26). After autologous transplant, thymopoiesis has been shown to be critical for the restoration of peripheral $CD4^+$ T cell population. This recovery encompasses the reconstitution of normal $CD4^+$ T cell numbers expressing a diverse TCR repertoire (26). Moreover, for HIV^+ patients on highly active antiretroviral therapy, thymic volume increase has been associated with better $CD4^+$ T cell recovery (27). The data presented here suggest that, when the number of T cells depends greatly on the proliferation of an extremely reduced set of peripheral T cells and on de novo thymic export, the infection of the thymus by mycobacteria may have, in humans, a relevant negative effect on the ability to control chronic slow progressing or latent infections.

In summary, we have shown here that during infection with *M. avium,* infected cells within the thymus induce tolerance specifically to mycobacterial antigens. To our knowledge, this is the first report showing induced central tolerance to an infecting pathogen. Knowing that the thymus is also a target organ for mycobacterial infections in humans, it is now important to evaluate to what extent the newly generated T cells from patients infected with *M. avium* or *M. tuberculosis* present an impaired ability to respond to mycobacterial antigens.

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A.
SPLEEN
LIVER
THYMUS
Log 10 CFU
Time post infection (wk)
B.
M. avium
DAPI
cortex
medulla
Merge
C.
M. avium
CD11c
F4/80
Merge
D.
M. avium
CD11c
F4/80
Merge
E.
M. avium
CD11c
Ep-Cam
Merge

Figure 1. Identification of *M. avium* infected cells in the thymus. (A). Representative kinetics of *M. avium* bacterial load in the spleen, liver and thymus. Each point represents the mean +SD CFU from 6 mice per group. (B-E) Representative thymic sections from *M. avium* infected mice (20 weeks post infection) stained with antibodies specific for mycobacteria (B-E), CD11c (C-E), F4/80 (C, D) and Ep-Cam (E). (B) The cortex can be distinguished from the medulla by the high density of nuclei stained with DAPI (blue) (transition of these regions marked with a dashed line). Mycobacteria (green) are mainly localized in the transition from the cortex to the medulla and in the medulla. (C) Bacteria (green) are mainly found within $CD11c^+$ (blue) $F4/80^+$ (red) cells. (D) Some of the infected cells are $CD11c^+F4/80^-$. (E) No bacteria (green) were detected in $EpCam^+$ cells (red). The inset shows bacilli within $CD11c^+$ cells in the vicinity of $EpCam^+$ cells. Scale bar represents 50μm (B, E) or 20μm (C, D).

Figure 2. T cells whose differentiation take place within infected thymi present impaired ability to fight the pathogen in peripheral organs. (A) Thymic lobes from TCR$\alpha^{-/-}$ mice were transplanted under the kidney capsule of nude mice (thymic donors were animals previously infected with *M. avium* or age-matched non-infected controls). Transplanted mice were infected with *M. avium* 3 days after surgery and sacrificed 9 weeks later. (B) Representative plots of the CD4 and CD8 staining in the blood at different time points after transplantation with non-infected (upper panels) or infected (lower panels) thymic lobes. WT (left upper panel) and nude (left lower panel) blood cells are also depicted. (C). Number of CD4$^+$ T cells (left panel) and CD8$^+$ T cells (right panel) in the spleen. (D) Representative histograms of the expression of CD62L (top panel) and CD44 (bottom panel) on CD4$^+$ T splenocytes. Each line is one representative mouse from each group: gray filled histogram corresponds to a WT mouse; empty histograms correspond to a nude mouse transplanted with infected (dotted line) or non-infected (solid line) thymic lobe. (E) Bacterial loads were assessed in liver and spleen 9 weeks after infection. Each column represents the mean +SD of the percentage of cells (C) or of the CFU (E) from 3 to 7 mice per group from 1 out of 3 independent experiments with similar results.

Figure 3. T cells differentiated within infected thymi show impaired ability to produce IFN-γ in response to *M. avium* antigens. (A) Splenocytes from WT and from nude mice transplanted with non-infected or infected thymi (right, middle and left panels) were stimulated *in vitro* with Con A, *M. avium* total extract (referred as *M. avium* in the figure) and with a panel of antigenic fractions prepared from the supernatant of the *M. avium* culture (Fr1 to Fr20) (details on the preparation in Fig. S2). IFN-γ concentration was assessed in the cell supernatants by ELISA. (B) Thymic lobes from TCRα$^{-/-}$ infected or non-infected mice were transplanted under the kidney capsule of nude recipient mice. Transplanted mice were infected 3 days later with *M. avium* and sacrificed after 9 weeks. Purified CD4^{+} T cells from these mice were adoptively transferred to TCRα$^{-/-}$ mice which were infected with *M. avium* 3 days later. (C) Splenocytes of TCRα$^{-/-}$ mice adoptively transferred with CD4^{+} T cells from nude mice transplanted with non-infected (left panel) or infected thymi (right panel) were stimulated with the same antigens described in A. Each bar represents the mean + SD of the IFN-γ concentration from 3-6 mice per group from 1 out of 3 independent experiments.

Figure 4. T cells differentiated within infected thymi respond *in vitro* to OVA upon immunization. Nude mice transplanted with thymi, as described previously, were immunized with OVA and sacrificed 1 week after the last immunization. The frequency of IFN-γ producing splenocytes, when stimulated with Ag85 (left panel) or with OVA (righ panel), was assessed by ELISPOT. Each bar represents the mean + SD of the number of IFN-γ producing cells from 4-5 mice per group.

Figure 5. T cells differentiated in infected thymi do not participate in the formation of granulomas. Liver section from mice transplanted with thymic lobes (as described in Fig. 2), and control mice, were analyzed 9 weeks post infection. (A) Representative haematoxylin-eosin staining showing 3 types of hepatic lesions: inflammatory infiltrates (left panel); poorly-organized granuloma (middle panel); well-organized granuloma (right panel). Each scale bar represents 50µm. (B) The percentage of each type of lesion was assessed. (C) Percentage of $iNOS^+$ lesions in the liver. Each bar represents the mean +SD from 5-9 mice per group. (D) Representative iNOS staining of liver lesions from WT, nude, nude transplanted with infected or non-infected thymic lobes (from left to right).

Figure S1. *M. avium* infection of the thymus does not lead to premature thymic atrophy but causes minor changes in cell populations at late time points. A. Number of thymocytes in infected and age-matched control mice. **B.** The percentage of the four main thymic populations (accordingly to the expression of CD4 and CD8 surface markers) between infected and age-matched control animals at different time points post infection. **C.** TCR rearrangement in the thymus assessed by the relative quantification of TREC. Briefly, genomic DNA was isolated from spleen and thymus using Trizol reagent. Quantification of sjTREC was performed by quantitative-RT-PCR (qRT-PCR) using the TCRA constant gene as endogenous reference [specific primers used were described elsewhere (Broers, A.E., J.P. Meijerink, J.J. van Dongen, S.J. Posthumus, B. Lowenberg, E. Braakman, and J.J. Cornelissen. 2002. Exp Hematol 30:745-750)]. qRT-PCR reactions were performed on a CFX96 Real Time System (BioRad) using QuantiTect SYBR Green RT-PCR reagent kit (Qiagen). All melting curves exhibited a single sharp peak. Each bar represents the average ± SD of the number (B) or percentage of cells (C) or relative amount of TREC from 3 to 6 mice per group. * $p<0.05$ and ** $p<0.005$.

Figure S2. Representative fractionation profile of *M. avium* culture supernatant proteins. *M. avium* antigenic fractions were prepared as specified elsewhere (Pais, T.F., J.F. Cunha, and R. Appelberg. 2000. Infect Immun 68:4805-4810). Briefly, *M. avium* from log-phase cultures were inoculated into Sauton medium (enriched with 0.5% sodium pyruvate and 0.5% glucose) and grown at 37°C without shaking. In the middle of the log-phase of growth (i.e. at day 10, as evaluated from the previous growth curves), cultures were centrifuged and the supernatants filter-sterilized for processing. After reducing the sample volume using an Amicon YM membrane (Millipore, molecular cut-off of 3000) protein was precipitated with 80% ammonium sulphate. Protein was recovered by ultracentrifugation and resuspended in apyrogenic PBS. 4mg of total protein was separated by SDS-PAGE (with a gradient of 10 to 20% of acrylamide) and the gel prepared for electroelution. Proteins were electroeluted into 2mM PBS in a whole gel eluter (BioRad) in a cold-room. The fractions were collected, quantified (by the Micro BCA method, from Pierce) and analysed in a silver-stained gradient SDS-PAGE (10 to 20% acrylamide).

Figure S3. Similar percentages of CD4$^+$ T cells in the spleen of TCRα$^{-/-}$ mice transferred with cells from the nude mice transplanted with non-infected or infected thymus. TCRα$^{-/-}$ mice were infected with *M. avium* and 16 weeks after were sacrificed and their thymuses collected for transplant under the kidney capsule of nude recipient mice. Thymic lobes of age-matched non-infected mice were also transplanted into another group of nude mice. Transplanted mice were then infected with *M. avium* and sacrificed 9 weeks later and their spleen CD4$^+$ T cells were purified and transferred to TCRα$^{-/-}$ mice. These mice were infected with *M. avium* and sacrificed 18 weeks later (depicted in Fig. 3). The percentage of CD4$^+$ T cells was assessed by FACs in the spleens of TCRα$^{-/-}$ mice that were adoptively transferred with cells from nude mice transplanted with infected or non-infected thymi.

3.2.

COMPLEMMENTARY INFORMATION: THE ROLE OF NEWLY DIFFERENTIATED T CELLS ON THE COURSE OF CHRONIC *M. AVIUM* INFECTION

The role of newly differentiated T cells on the course of chronic *M. avium* infection

BACKGROUND

The immune response to the *M. avium* infection is a chronic process and constantly dependent on the activity of T cells. For this reason, we assessed the role of the thymic output on the outcome of the infection. Few studies have assessed the importance of thymic output during mycobacterial infections (1-3). None has attributed a significant role to the newly differentiated T cells on the progression of the infection. However, those studies were performed for a relatively short period of time taking into consideration the long course of mycobacterial infections. Therefore, we have extended those experiments to longer periods during chronic *M. avium* infection.

EXPERIMENTAL PROCEDURES

Mice

Eight to 10 weeks-old C57BL/6 female mice (Harlan, Barcelona, Spain) were used, in accordance with National and European Union guidelines for the care and handling of laboratory animals. Mice were maintained in conventional conditions, under 12 hours light-cycles and were given *ad libitum* chow and sterile tap water.

Thymectomy

Mice were anaesthetised with xylazine and ketamine [200μg xylazine hydrochloride (Bayer) and 100mg ketamine hydrochloride (Merial) administered intravenously]. The thymus was removed by suction through an incision made in the upper anterior part of the chest. Sham-thymectomized mice underwent surgery however, thymi were not removed. Complete removal of the thymus was confirmed for each mouse at sacrifice.

Bacterial strain and experimental infection

Mice were infected one week after thymectomy with *M. avium* strain 2447 (smooth transparent variant; intermediate virulence). Infection was performed intravenously through the lateral vein with 10^6 colony forming units (CFU). At specific time points, mice were sacrificed and the bacterial load in the organs was determined by plating serial dilutions of tissue homogenates onto 7H10.

Single cell suspensions and flow cytometry analysis

Spleen single cell suspensions were prepared by gentle disruption of the organs between two notched glass slides. Cells were washed and resuspended in DMEM supplemented with 10% heat inactivated FCS, 10mM HEPES, 1mM sodium-pyruvate, 2mM L-glutamine, 50μg/ml streptomycin and 50U/ml penicillin. Splenocyte suspensions were further depleted of red blood cells by treatment with ACK lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, pH 7.2) for 5 min at room temperature, washed and resuspended in supplemented DMEM. Cells were stained for flow cytometry by incubating for 20 min on ice with combinations of the following antibodies: anti-CD3 FITC (145-2C11), anti-CD4 PeCy5.5 (RM4-5), anti-CD8 PE or FITC (53-6.7), anti-CD44 PE (IM7), anti-CD62L FITC (Mel14), anti-CD19 Pe (6D5) and anti-CD11c FITC (N418) (all from BD Pharmingen, San Diego CA, USA). Cell acquisition was performed on a FACSort flow cytometer (Becton

Dickinson, NJ, USA) equipped with Cell Quest software. Dead cells, identified by their light-scatter characteristics, were excluded from analysis.

Detection of IFN-γ in the serum

To obtain serum, mice were anesthetized before sacrifice with isoflurane and retro-orbital bleeding was performed. Blood was allowed to clot and the serum was collected after centrifugation. Samples were frozen at -70ºC until use. Quantification of IFN-γ was performed by a two-side sandwich ELISA with anti-IFN-γ-specific affinity-purified mouse antibodies (R46-A2 as capture and biotinylated AN-18 as detecting antibodies). Standard curves were generated with known amounts of recombinant IFN-γ. The sensitivity of the assay was 20pg/mL.

Statistical analysis

Differences between means of experimental groups were analyzed by two-tailed Student's t-test. Differences with a P value of 0.05 or less were considered significant. Significance was referred as * when P≤0.05, as ** when P≤0.005 or as *** when P≤0.001. All values are represented as mean + SD.

RESULTS

Thymectomy has no effect on the outcome of systemic *M. avium* infection

To assess the importance of thymic output during *M. avium* infection we thymectomized adult mice prior to infection. Control mice underwent surgery but thymi were not removed. Spleen cell subsets were analysed and the bacterial load in spleen and liver quantified. Because we are studying a chronic infection, time points as long as 31 weeks post infection were assessed.

When comparing the populations present in the spleen of both groups no major differences are observed on the percentages of macrophages in the course of infection. B cells, on the other hand, are consistently present in higher percentages in thymectomized mice unlike T cells that are present in lower levels in this group (Fig. 1A). The decrease in the percentage of T cells in thymectomized mice is due to a decrease in $CD4^+$ (in all time points assessed) and $CD8^+$ T cells (only from 12 weeks on) (Fig. 1B). As $CD4^+$ T lymphocytes are the main players in the immune response to mycobacterial infections (1, 4-6), we assessed the activation profile of these cells by analysing the cell surface expression of CD44 and CD62L. From 12 weeks on an increased percentage of $CD62L^-$ cells is observed in splenocytes from thymectomized mice (Fig. 1C and D). In the same group, an increased expression of $CD44^{high}$ cells is detected 24 weeks post infection (Fig. 1C and D). Altogether, these data shows that upon infection, thymectomized mice, in comparison to sham-thymectomized mice, have lower percentages of T cells and a concomitant increased percentage of $CD4^+$ cells with an activated profile.

To evaluate the impact of thymectomy on the outcome of *M. avium* infection, we have quantified the bacterial load in spleen and liver. No major differences are observed in the bacterial loads of the two groups during the course of infection except for a transient increase in the spleen bacterial load of thymectomized mice at 15 (experiment #1) or 12 (experiment #2) weeks post infection, depending on the experiment (Fig. 2). A significant transient increase in the bacterial load in the liver of thymectomized mice is also observed at 15 weeks post infection, but only in one of the experiments. However, these differences were not confirmed for longer time points. When the concentration of IFN-γ was assessed in the serum, no differences are observed between the two groups (Fig. 3).

Figure 1. Thymectomized mice, in comparison to sham-thymectomized mice, have a decreased proportion of CD4$^+$ T cells and simultaneously an increased percentage of CD4$^+$ T cells with an activated phenotype. Infected thymectomized and sham-thymectomized mice were sacrificed at 4, 12 and 24 weeks post infection. Spleen cell subsets were assessed by flow cytometry. **A.** Percentages of macrophages (CD11b), B cells (CD19) and T cells (CD3) in the spleen. **B.** Percentage of CD4$^+$ and CD8$^+$ T cells in each group. **C.** Percentage of CD4$^+$ cells with an activated profile assessed by the lack of CD62L expression (CD62L$^-$) or up-regulation of CD44 cell surface expression (CD44^{high}). **D.** Representative histograms of CD62L (upper panel) or CD44 (lower panel) surface expression in thymectomized and in sham-thymectomized mice 24 weeks post infection. Each line refers to one representative mouse from each group: black open histograms correspond to a sham-thymectomized mouse and gray filled histogram to a thymectomized mouse. In A, B and C, each bar represents the mean + SD from 6 to 8 mice per group from one of two independent experiments with similar results.

Figure 2. Thymectomy has no effect on the *M. avium* bacterial load. Mice were thymectomized and infected with *M. avium* one week later. Control mice underwent surgery but thymi were not removed. Bacterial load in the spleen and liver from two independent experiments are depicted. Each bar represents the mean + SD from 5 to 6 mice per group in experiment 1 or from 6 to 8 mice per group in experiment 2.

Figure 3. Serum IFN-γ levels in thymectomized mice are similar to sham-thymectomized mice. Quantification of the serum IFN-γ levels was performed by ELISA for thymectomized mice and sham-thymectomized at 4 and 24 weeks post infection. Each bar represents the mean + SD from 5 to 6 mice per group from a single experiment.

DISCUSSION

Our results show that newly generated T cells make an important contribution for the peripheral T cell number, however in the course of infection, these cells have no role in the control of the bacterial load even for periods as long as 31 weeks post infection.

Upon thymectomy, we can observe a clear decrease in the percentages of T cells, mainly $CD4^+$ T cells and, only later, of $CD8^+$ T cells. Moreover, an increased percentage of $CD4^+$T cells exhibited an activation profile, assessed by the low expression of CD62L and high expression of CD44. These data are in agreement with a study where the thymic output was abolished by conditional ablation of Rag2 (recombination activating gene), in the absence of infection. This report describes a marked decrease in the number of $CD4^+$ T cells early upon cessation of thymic output whereas $CD8^+$ T cells numbers only decrease some weeks later (7). Moreover, a marked decrease in the naive $CD4^+$ T cell pool (as assessed by $CD44^{low}CD25^-$) is observed though the memory pool ($CD44^{high}CD25^-$) was not affected up until 14 weeks post-ablation of Rag2 (7).

Thymectomy is often performed in infants during cardiac surgery. In these situations, it has been described that later in live, the numbers of T cells are moderately decreased in comparison to age-matched controls (8-12). Moreover, a study performed in children that were thymectomized 15 years earlier has shown that these individuals exhibited a decreased naive T cell pool (12). Based on this, and on TRECs analysis, the authors have suggested that T cell homeostasis in thymectomized individuals is maintained mainly by extrathymic expansion of existing naive T cells in the periphery to compensate for the lack thymic output (12). It is interesting to note however, that thymectomized individuals do not exhibit increased susceptibility to infections (even when opportunistic infections were assessed) in comparison to non-thymectomized individuals (8, 10-13). However, no studies have been published regarding the immune response of these individuals in response to chronic infections.

Our results extend and are in agreement with previously reported data on the outcome of *M. avium* (using the same intermediate-virulence strain) infection upon thymectomy (1, 2). These studies have assessed the bacterial load in thymectomized and control mice and no differences were detected on the bacterial load up until 6 (2) or 13 weeks (1) after infection. Florido *et al.* have also evaluated the role of thymic output during infection with a high-virulence strain of *M. avium* (strain 25291). In this case, it was observed that 6 weeks post infection thymectomized mice have increased bacterial load in spleen and liver in comparison to non-thymectomized mice. Moreover, they have also shown that infection with this strain of *M. avium* was responsible for a severe depletion of peripheral T cells in

unmanipulated mice (2). Therefore, these observations emphasize the importance of newly generated T cells on the outcome of the infection in situations where lymphopenia is induced.

The lack of differences in the bacterial load in thymectomized *versus* sham-thymectomized mice indicates that, when thymectomy is performed, both the repertoire and the size of the peripheral T cell pool is enough to control the bacterial growth. Therefore, the already existing peripheral T cells are sufficient to mount and maintain an effective immune response. In agreement, Winslow *et al.* have thymectomized mice and infected with *M. tuberculosis*. 50 days post infection, $CD4^+$ T cells specific for the ESAT-6_{1-20} peptide were detected at similar levels in thymectomized and control mice. The authors suggested that the ESAT-6_{1-20}-specific response present in the lungs was probably derived entirely from the peripheral T cells pool (3).

In summary, our data suggest that the thymic output has no role over the course of *M. avium* chronic infections probably because the peripheral T cells pool is sufficient to control the bacterial growth. Nevertheless, one should also consider that T cells exported from an *M. avium* infected thymus are not able to mount a protective immune response (Nobrega *et al.* submitted for publication). Considering this, it is likely that the thymic output in sham-thymectomized mice does not contribute to an effective immune response.

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CHAPTER 4

ANTIBIOTIC TREATMENT HAS A REDUCED EFFECT ON *MYCOBACTERIUM AVIUM* WITHIN THE THYMUS

Antibiotic treatment has a reduced effect on *Mycobacterium avium* within the thymus

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ABSTRACT

We have recently demonstrated that the thymus becomes progressively colonized in mice infected with different species of mycobacteria. Moreover, several case reports showed that the thymus is also, in humans, a target organ for mycobacterial dissemination. Of notice, in one of these human cases an increased resistance of the mycobacteria to the antibiotics was reported. This observation prompted us to investigate the efficacy of the antibiotic treatment against mycobacteria within the thymus compared to bacteria present in other organs. To do so, we used the established model of *Mycobacterium avium* infection and assessed the efficacy of the antibiotic treatment for the bacteria present in the thymus and in other organs. Herein we show that treatment administered in the drinking water leads to a decrease in the bacterial load in spleen, liver and lung, although within the thymus the bacterial load remains unaltered throughout the treatment. When a similar dose of antibiotic is delivered by gavage a more drastic effect is observed in the bacterial load in all organs assessed, including the thymus. Nonetheless, it is still clear that, even in this situation, the decrease in the bacterial load in the thymus is not as strong as in other organs. The reasons accounting for the observed decreased susceptibility to antibiotics of *M. avium* within the thymus are discussed as well as the potential impact of these observations on the development of new therapeutic strategies.

INTRODUCTION

Treatment of mycobacterial infections is quite complex. Due to the nature of the mycobacterial cell wall and the intracellular location of the *bacilli* (1, 2) multidrug therapy must be administered and continued for long periods of time (6 to 12 months) to achieve clinical cure (2, 3). In the case of infections with the *Mycobacterium avium* complex (MAC), several factors render the antibiotic treatment extremely difficult. One of the main problems is the wide diversity of antibiotic susceptibility among MAC isolates. This, coupled with the fact that a substantial number of patients infected with the acquired immunodeficiency virus (AIDS) are infected with more than a single MAC isolate definitely contributes to the lack of success in treating MAC infections (1). Moreover, treatment of MAC infections with antibiotics known to be useful for *Mycobacterium tuberculosis* infections gives rise to inconsistent results; most first-line anti-*M. tuberculosis* drugs have 10 to 100 times less *in vitro* activity against MAC isolates than against *M. tuberculosis*. Another concern when studying antibiotic efficacy against MAC infections is the low reproducibility of the *in vitro versus in vivo* results. Until the late eighties, one of the main concerns in the development of drug therapies for MAC infections was the lack of drugs with low toxicity and good efficacy *in vivo* (3). The development of macrolides such as clarithromycin and azythromycin enabled a major advance in the therapy of MAC infections (4-11). However, monotherapy with any of these macrolides often results in the emergence of drug-resistant species (12, 13). Complementation of the therapy with other drugs decreases the incidence of drug-resistant *bacilli*. Nevertheless, drug-resistant bacteria may still emerge during multidrug therapy (14).

Administration of combined therapy is important not only to minimize the emergence of drug-resistant microorganisms (3) but also to increase the microbicidal activity of agents which have only modest activity when used individually (15, 16). Antibiotic combination of clarithromycin, ethambutol and rifampicin is one of the most widely used treatments against MAC infections (3, 9, 17-20).

Ethambutol inhibits the synthesis of arabinogalactan (21, 22) and of lipoarabinomannan (21) and also inhibits the transfer of mycolic acids into the cell wall of the mycobacteria (23). This compromises the cell wall biosynthesis and renders mycobacteria cell wall more permeable (24) and hence, enhance the effect of other drugs, such as clarithromycin and rifampicin (17). Both clarithromycin and rifampicin interfere, though at different levels, with protein synthesis (25-27). However, clarithromycin has increased efficacy against MAC due to its pharmacokinetic properties (because it is stable in acidic environment it might remain active for longer periods within the acidic environment of the phagolysosome) (5,

28, 29) and due to its ability to achieve high concentrations in tissues and inside macrophages (4, 5, 17, 30, 31). Rastogi *et al.* have illustrated, in a very simple way, the synergistic effect of clarithromycin, ethambutol and rifampicin, *in vitro*. By electron microscopy, they have shown that clarithromycin-treated bacteria exhibit disorganized outer wall layer and cytoplasmic membrane that results in the formation of large vacuoles inside the cytoplasm, with solubilisation of ribosomal structures and consequent plasmolysis. Association of clarithromycin to ethambutol and rifampicin results in more drastic alterations than the ones seen with any of the drugs alone, leading to the removal of the bacterial outer layer, homogenization of cytoplasm, complete cell lysis and formation of ghosts (17).

To date, several case reports on thymic infection with mycobacteria have been published (32-40). Among them, the report by Sacco *et al.* describes a case of persistent thymic tuberculosis upon anti-mycobacterial therapy (38). The mechanism underlying the observed resistance of the bacteria within the thymus was not explored. No other studies assessing the effect of anti-mycobacterial therapy on the *bacilli* within the thymus were published.

We have previously reported, using the mouse model of infection, that the thymus is a target organ for infection with *M. avium*, *Mycobacterium bovis* bacillus Calmete-Guérin (BCG) (inoculated intravenously) and *Mycobacterium tuberculosis* (via aerosol inhalation) (41). We have observed also that the bacterial plateau within the thymus is achieved at later time points in comparison to the spleen and liver. Moreover, despite the large accumulation of infected macrophages within the thymus, organized granulomas were not observed within this organ (41). These observations suggest that the interaction between the *bacilli* and the host differs within the thymus when compared to other organs. Taking all these observations into consideration we decided to explore the impact of chemotherapy on the control of the bacterial load within the thymus. We show that, using a combination of ethambutol, clarithromycin and rifampicin, *bacilli* within the thymus are more difficult to eliminate in comparison to bacteria within the spleen, liver or lung. Moreover, when clarithromycin alone or ethambutol plus rifampicin are administered a minor effect is observed in the bacterial load in the thymus compared to untreated mice, despite the clear bactericidal and/or bacteriostatic effected observed in the spleen, liver and lung.

EXPERIMENTAL PROCEDURES

Mice

All experiments were conducted using 8 to 10 week old C57BL/6 female mice (Harlan, Barcelona, Spain), in accordance with National and European Union guidelines for the care and handling of laboratory animals. Mice were maintained in specific pathogen free (SPF) conditions, under 12 hours light-cycles and were given *ad libitum* chow and sterile tap water. In some experiments, specified in the results section, antibiotics were added in the drinking water.

Bacterial strain and experimental infection

Infection with *M. avium* strain 2447 (smooth transparent variant with intermediate virulence) was performed intravenously through the lateral vein with 10^6 colony forming units (CFU). At specific time points, mice were sacrificed and the bacterial load in the organs was determined by plating serial dilutions of tissue homogenates onto 7H10 as previously described (41). The detection limit for CFU determination varied depending on the organ assessed: 0.3log CFU for spleen, lung and thymus and 1.6log CFU for liver.

Antimycobacterial therapy

Chemotherapy for *M. avium* was initiated eight or nine weeks after infection (as specified in results section) and consisted of a cocktail of rifampicin (Sigma, St. Louis, MO, USA), clarithromycin (Abbott Laboratories, Abbott Park, IL, USA) and ethambutol (Sigma). When antibiotics were administered in the drinking water, the combination of antibiotics (0.4mg/mL rifampicin, 1.94mg/mL clarithromycin and 0.25mg/mL ethambutol) was available to mice during the whole period of treatment; mice drunk, on average, 3mL of the antibiotic cocktail per day. When administered by gavage, 0.3mL of the cocktail (containing 4.67mg/mL rifampicin, 22.5mg/mL clarithromycin and 2.92mg/mL ethambutol) was given 6 days a week; control mice received the same amount of sterile tap water by gavage. It should be stressed that, on average, mice received the same dose of antibiotic per week, whether the administration was done by gavage or in the drinking water. In order to avoid the effect of antibiotics on the bacterial growth in the plate, the administration of antibiotics was stopped 48 hours before mice where sacrificed.

Determination of the decimal reduction time (D-value)

The resistance of the *bacilli* to the antibiotic treatment was characterized in terms of decimal reduction time, D-value, which is the exposure time required, under specified conditions, to cause 90% reduction (or 1log reduction) of the initial population. The D-value was determined by the equation: $D = t \times [\log CFU(t_i) - \log CFU(t_f)]^{-1}$, where D is the D-value (weeks) at specified conditions. $CFU(t_i)$ and $CFU(t_f)$ are the average bacterial loads at the beginning of the treatment (t_i) or of the survival population after an exposure time (t_f), respectively. t (weeks) = (t_f-t_i) is the exposure time to antibiotic treatment.

Statistical analysis

Comparisons between groups were performed using a Student's t-test. Differences were considered statistically significant when the P value was 0.05, or less, and were represented by *. All values are represented as mean +/- SD.

RESULTS

Chemotherapy is less efficient at eliminating bacteria within the thymus than within other organs

To assess the efficacy of the antibiotics within the thymus we infected mice intravenously with an intermediate-virulence strain of *M. avium* and 8 to 9 weeks later an antibiotic regimen was started. The treatment consisted of a combination of three different antibiotics: clarithromycin, ethambutol and rifampicin. Since oral administration of antibiotics can be performed either by adding them in the drinking water of by gavage, leading to different outcomes, we tested both modes of administration.

As it can be observed in Fig. 1, anti-mycobacterial treatment, administered in the drinking water (Fig. 1A) or by gavage (Fig. 1B), efficiently decrease the bacterial load in spleen, liver and lung. Antibiotic administration in the drinking water induces bacteriostasis on the *bacilli* residing within the thymus: the bacterial load is maintained constant during the whole treatment (Fig. 1A). It is interesting to note that, after 33 weeks of antibiotic treatment in the drinking water a large difference in the bacterial load between treated and untreated mice in the spleen (3.7log CFU less), liver or lung (3.9log CFU less for both) is observed whereas in the thymus that difference is of 1log CFU only.

Although mice treated either by gavage or with antibiotics in the drinking water receive a similar dose of antibiotics per week, administration by gavage induces a stronger and faster decrease of the bacterial loads (Fig. 1B). When D-values are calculated it is clear that less time is required for a 90% decrease on the bacterial load in the organs of mice treated by gavage in comparison to mice treated with antibiotics in the drinking water (Table 1). Of notice, the bacterial load in the thymus also decreases upon treatment by gavage, as opposed to the bacteriostatic effect observed during administration of the antibiotics in the drinking water, but again the efficacy of treatment in the thymus is reduced compared with the effect on bacteria within other organs (Fig. 1B). For the antibiotics given by gavage to induce a reduction of 1log CFU in the thymus more than twice the time of treatment (4.9 weeks) is required in comparison to the other organs (1.5-1.8 weeks) (Table I). In fact, when the treatment is initiated, the bacterial loads in the lung and thymus are similar; however, 4 weeks of treatment are enough to eliminate the *bacilli* from the lung whereas in the thymus this treatment results in a modest reduction of 0.8log CFU (a difference that is not statistically significant).

Figure 1. Reduced efficacy of antibiotic to eliminate *M. avium* within the thymus when compared with *bacilli* in the spleen, liver or lung. Mice were infected intravenously with *M. avium* and, 8 or 9 weeks later, an antibiotic regimen, administered in the drinking water (A) or by gavage (B), was started. Bacterial load was assessed in the spleen, liver, lung and thymus. Control mice were left untreated (A) or were given water by gavage (B). Each point represents the mean + SD of 6 mice per group, from one representative experiment out of three (A) or from a single experiment (B). Statistical analysis was performed by comparing each group's bacterial load with the bacterial load in the beginning of the treatment. Dashed lines stand for the detection limit.

Table 1. D-values for *M. avium* in the different organs upon administration of antibiotics in the drinking water and by gavage.

Route of antibiotic administration	D- values (weeks)	
	in the drinking water [(1)]	by gavage [(2)]
Spleen	12,8	1,8
Liver	7,6	1,6
Lung	14,4	1,5
Thymus	[(*)]	4,9

[(1)] It was assumed that a linear decrease on the bacterial loads was observed over the entire period of treatment; for this reason D-value was calculates from 9 to 42 weeks post infection. [(2)] Calculations were performed from 8 to 12 weeks post infection because afterwards no bacilli were detected in the lung. [(*)] D-value can not be calculated for the bacteria within the thymus upon administration of antibiotics in the drinking water because therapy, in this case, has a bacteriostatic effect.

The use of clarithromycin, in combination with both ethambutol and rifampicin is essential to achieve bacteriostasis within the thymus

Of the three drugs assessed, clarithromycin is reported as the most efficient in the control of *M. avium* growth (5, 8-10). However, because this molecule is relatively large (MW 748), it may be prevented from entering the thymus due to the presence of the blood-thymus barrier (BTB). Therefore, we hypothesized if the decreased efficacy of the antibiotic on the *bacilli* within the thymus was due to the reduced entrance of clarithromycin into the thymus. If this was the case, the bacteriostatic effect observed for the bacteria present in the thymus would result from the effect of ethambutol and/or rifampicin. To address this issue we analysed whether the lack of this drug in the antibiotic cocktail would also result in thymic bacteriostasis. We infected mice and, 8 weeks later, antibiotic treatment administered in the drinking water was started with three different combinations of antibiotics: i) clarithromycin; ii) ethambutol and rifampicin; iii) clarithromycin, ethambutol and rifampicin. Administration in the drinking water was chosen because this was the model where a more pronounce difference in the effect of the antibiotic within the thymus was observed in comparison to other organs.

As can be observed in Fig.2, the combination of the three antibiotics is the most efficient in reducing the bacterial loads in spleen, liver and lung. Nevertheless, clarithromycin alone or ethambutol plus rifampicin also have a bactericidal effect in these organs (except for ethambutol plus rifampicin in the lung at 16 weeks post-treatment). In contrast, in the thymus the three drugs together are necessary to achieve bacteriostasis and ethambutol plus rifampicin or clarithromycin alone have only a minor effect on the bacterial loads in comparison to untreated mice.

Figure 2. The combination of clarithromycin, ethambutol and rifampicin is necessary to induce bacteriostasis in the thymus. Mice were infected intravenously with *M. avium* and 8 weeks later an antibiotic regimen, administered in the drinking water, was started. Three different combinations of the drugs were used. Control mice were left untreated. Bacterial load was assessed in the spleen, liver, lung and thymus by the time chemotherapy was started, 8 and 16 weeks after. Each point represents the mean +/- SD of 6 mice per group from a single experiment. Statistical analysis was performed by comparing each group's bacterial load with the bacterial load in the beginning of the treatment. EMB: ethambutol, RFP: rifampicin; CLA: clarithromycin.

DISCUSSION

In the present report, we show that *M. avium* load within the thymus is less affected by the treatment with a cocktail of antibiotics (composed of clarithromycin, ethambutol and rifampicin) in comparison to bacteria in the other organs assessed. This difference is more evident when the antibiotic combination is administered in the drinking water than by gavage. Moreover, whereas in the spleen, liver and lung ethambutol plus rifampicin or clarithromycin alone have a bacteriostatic or bactericidal effect, in the thymus these combinations are only responsible for a slight delay on the increase of the bacterial load. The decreased ability for the antibiotics to eliminate the *bacilli* within the thymus is discussed herein as well as the potential impact of these observations on the treatment of *M. avium* infections.

Our results clearly show that administration of clarithromycin, ethambutol and rifampicin has a weaker effect on the bacterial load in the thymus in comparison to bacteria within the other organs studied. Administration of antibiotics in the drinking water induces bacteriostasis in the thymus as opposed to the bactericidal effect in the spleen, liver or lung. In contrast, when the antibiotics were administered by gavage a bactericidal effect was also observed in the thymus although this effect was not as severe as the one observed for the other organs. It should be stressed that by the time treatment by gavage was initiated the bacterial loads in the thymus and lung were similar; however, upon 4 weeks of treatment no *bacilli* were detected in the lung whereas in the thymus a non-significant decrease in the bacterial load was observed. This observation clearly suggests that the efficacy of antibiotic treatment is reduced for bacteria within the thymus in comparison to bacteria in other organs even when administration by gavage is performed.

Administration of the antibiotics in the drinking water or by gavage leads to different outcomes on the bacterial load of all organs studied. Gavage induces a more pronounced decrease in the bacterial loads, as determined by D-values. In contrast to administration of the antibiotics in the drinking water, administration by gavage leads to undetectable levels of bacterial loads in the lung and liver at 4 and 12 weeks of treatment, respectively. Despite the different effect of the antibiotic on the bacterial load, depending on the mode of administration, in both situations, antibiotics are less efficient for *bacilli* within the thymus. This effect is more obvious upon antibiotic administration in the drinking water, which induces bacteriostasis. Administration of antibiotics by gavage has a bactericidal effect in the thymus though much less severe than the effect observed on the other organs assessed (by the end of the treatment a decrease of more than 3,5log CFU was observed for spleen and liver as opposed to the 2log CFU decrease in the thymus). The different outcome of the antibiotic treatment depending on the mode of administration may

be related to different concentrations of antibiotic attained in the serum in each situation and, as a consequence, within each organ. To our knowledge, studies assessing the outcome of different modes of oral antibiotic administration on the mycobacterial burden have not been performed. In a different context, a few studies have compared gavage and administration in the diet of drugs by evaluating the drug availability in the serum, or in specific tissues, and by following pharmacodynamic biomarkers (42-44). These studies were unanimous in showing that gavage leads to higher peaks of drug concentration in the serum in comparison to administration in the diet. These levels were transient and decreased to levels similar to, or below, the ones observed in animals receiving drugs administered in the diet. Due to the constant input of drug during administration in the diet, the absorption, distribution and elimination phases were occurring simultaneously resulting in less variable levels of drug throughout the study. The effect of the different modes of administration on the pharmacodynamic biomarkers was dependent on several factors inherent to the drug such as its half-life, absorption or mechanism of action (42-44).

Studies that assess the *in vivo* efficacy of antibiotic treatment of mycobacterial infections often use as a read-out the effect on the bacterial load in the spleen, liver or lung (5, 15, 45, 46). Other organs are usually not taken into consideration. However, it has been widely shown that when organs such as the brain are considered, the antibiotic access to the tissue is, in most situations, a challenging process. This is largely attributed to the blood-brain barrier (BBB) that prevents the entrance of a great proportion of circulating macromolecules into the brain (47, 48). The thymus has also a blood barrier, the blood-thymus barrier (BTB) (49). Although much less studied than the BBB, it is known that the BTB is only present in the cortex of the thymus. While the thymic medulla is completely permeable, macromolecules that cross the medullary venules have a limited distribution in the thymic parenchyma because macrophages ingest and retain much of these molecules. The distribution of macromolecules within the thymus was shown to be dependent on its molecular weight and dose (49). Our data suggests that the bacteriostatic effect observed when the antibiotic cocktail is administered in the drinking water is not related to the lack of entry of clarithromycin (one of the largest and more efficient molecules on *M. avium* growth tested) into the thymus due to the BTB. Omitting clarithromycin from the cocktail lead to a minor delay in the bacterial growth in the thymus in contrast to the bactericidal effect in the spleen, liver or lung. Nevertheless, the observation that neither clarithromycin alone or ethambutol plus rifampicin were able to induce, at least, bacteriostasis in the thymus was quite surprising. This indicates that, as

opposed to the other organs assessed, only multidrug therapy is able to exert an effect on *M. avium* in the thymus, even if that effect is merely bacteriostatic.

The decreased effect of the antibiotic on the bacteria within the thymus might result from an altered metabolic activity of the *bacilli* within this organ that would render *M. avium* less susceptible to antibiotics. Of notice, Archuleta *et al.* have shown that *M. avium* cultures may enter a state of metabolic dormancy in situations of nutrient deprivation (50). When these cultures were tested for drug susceptibility it was shown that starved *bacilli* were more resistant to pyrazinamide and isoniazid in comparison to “non-starved” *bacilli* though the susceptibility to kanamycin was similar in both groups (50). Moreover, they have also suggested that recovery from this metabolic dormant state could be achieved within hours; in this situation, bacteria return to exponential growth phase and regain antibiotic sensitivity (50). However, this state of metabolic-dormancy of *M. avium* was never shown to exist in cell cultures or *in vivo*.

In summary, this report stresses the need to consider the thymus during antibiotic efficacy evaluations. Herein we present data that indicate that antibiotic treatment is less efficient on *bacilli* within the thymus than on bacteria in the other organs assessed. For this reason, the thymus may be regarded as a potential reservoir of bacteria upon cessation of antibiotic treatment. Because the thymus is where most of the T cell differentiation takes place, the persistent presence of *M. avium* within the thymus, even after being eliminated from other organs, may have deleterious effects on the differentiation and repertoire of the generated T cells.

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CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1. DISCUSSION

One of the main characteristics of the infections caused by *M. avium* and *M. tuberculosis* is the fact that they are responsible for chronic disease. To deal with these bacteria, the host has to rely on an effective immune system that depends, to a large extent, on T cells. These cells differentiate within the thymus and their repertoire is strongly influenced by the antigens they encounter in this organ during differentiation. Having this in consideration, and the fact that several case reports have shown that the thymus is a target organ for mycobacterial infections in humans, several questions arised and were addressed in this work. Thereby, the main goals of this thesis were: i) to characterize the kinetics of thymic infection, in the murine model, upon challenge with different mycobacterial species; ii) to assess the impact of such infection on the differentiation of newly generated T cells; iii) to study the impact of anti-mycobacterial therapy on the bacteria within the thymus. Here we describe that mice infected with *M. avium*, BCG or *M. tuberculosis* have their thymi progressively infected. The presence of *M. avium* within the thymus does not impair the production of new T cells. However, infection of the thymus by *M. avium* leads to the generation of new T cells that are tolerant specifically to mycobacterial antigens. When we assess the efficacy of antibiotic treatment on the thymic bacterial load, we observe that *bacilli* within the thymus are more difficult to eliminate in comparison to the ones in the spleen, liver or lung. The results obtained throughout this work are next integrated and the consequences of these observations on the progression of the immune response to mycobacterial infections and on the establishment of persistent infections are discussed. Moreover, new experiments are proposed to further dissect the findings presented here.

Upon infection, mycobacteria disseminate to the thymus

The thymus is a target organ for HIV infection in humans (1-7) as well as in experimental models such as the SCID-hu system (8-10). Infection of non-human primates with SIV also results in thymic colonization (11-13). In other experimental models, thymic infection is also observed as, for example, upon infection with measles virus (14), murine leukemia virus (15, 16), *T. cruzi* (17), *P. berghei* (18), *P. brasiliensis* (19) or *F. tularensis* (20).

Regarding thymic infection with mycobacteria, a few human case reports have been published (21-29). The presence of caseating granulomas was a constant in all case reports (21-29). In some reports, the infection was confirmed either by culture, acid-fast staining and/or by PCR (23-25, 28). In the late sixties Mauss studied thymic infection with *M. tuberculosis* in the murine model; however the use of extremely high bacterial doses that were lethal in a few weeks were not suitable for assessing the chronic nature of the

mycobacterial infections (30, 31). We now revisited this subject using bacterial inoculations that allow the animals to develop chronic disease. We show that upon aerosol inhalation of *M. tuberculosis* or intravenous infection with *M. avium* or BCG the thymus becomes infected (32). Thymic colonization takes place in a progressive way. On the day following inoculation with *M. avium*, very few bacteria are found in this organ*;* in the case of *M. tuberculosis* or BCG infection, no *bacilli* are detected in the thymus at this time point. Bacterial load increases up to 10^6 CFU, 16 weeks after infection with *M. avium,* and almost 10^4 CFU upon *M. tuberculosis* or BCG infection. Of notice, this progressive infection of the thymus is not exclusive for mycobacterial infections as the same was observed upon intraperitoneal infection with *P. berghei* (18).

How do the bacteria reach the thymus upon infection is still an open question. Both dissemination of the *bacilli* within phagocytic cells or as free bacteria in the blood can be envisaged and these mechanisms are not mutually exclusive. On day one after intravenous infection, although all animals have high numbers of viable *bacilli* within the liver, spleen and lung, about 20% of the animals show no thymic infection. Moreover, among the ones that reveal thymic infection, very few *bacilli* are detected (around 60% of the animals have from 1 to 3 viable bacteria in the thymus). A few weeks later, all mice have *bacilli* within the thymus. As bacteria are extremely scarce in the blood after the first day of infection, it is possible that other mechanism, distinct from the simple dissemination of the bacteria free in the bloodstream, contribute to continuous thymic infection after intravenous infection; this is the most probable case for aerogenic infection.

Dissemination of mycobacteria from the lung to the draining LN has been shown to occur within DCs. In these studies, Wolf *et al.* and Humphreys *et al.* have used *M. tuberculosis* and BCG tagged with GFP, respectively (33, 34). They have performed a temporal and quantitative characterization, by flow cytometry, of the cells harbouring mycobacteria tagged with GFP following aerogenic (in the case of *M. tuberculosis*) or intranasal infection (in the case of BCG). They showed that the first cells found infected in the LN were DCs (33, 34). Moreover, dissemination of *M. tuberculosis* to the LN was reduced in mice lacking CCL19 and CCL21, which are chemokines on the lymphatic endothelium required for migration of mature DCs.

Peripheral DCs can also migrate from the periphery into the thymus (35-37). Yet, DC migration into the thymus seems to be mainly restricted to immature DCs. Bonasio *et al.* have shown that DCs stimulated overnight with LPS (referred as mature DCs), when transferred into WT mice, migrate in lower numbers to the thymus in comparison to unstimulated DCs (immature DCs) (35). More recently, Li *et al.* have shown that upon entering the thymus, conventional DCs up-regulate CD80 and CD86 as well as CD11c

and MHC class II; at this time, they proliferate extensively and differentiate into mature DCs (37). Within the thymus, DCs with extra-thymic origin can participate in negative selection of thymocytes (35-39) as well as in the generation of regulatory T cells (36). We observe infected DCs within the thymus by immunofluorescence staining (as discussed later). Due to the key role played by peripheral DCs in the differentiation process of thymocytes it would be interesting to assess whether this cell subset is involved in disseminating mycobacteria into the thymus or if these cells become infected within the thymus. To test this hypothesis, the use of mycobacteria-tagged with GFP could be a good starting point to assess the cell subset that is first infected in the thymus, in a similar fashion to what has been published before for assessing *M. tuberculosis* and BCG dissemination to the draining LN. Nevertheless, additional studies would be required where blockage of cell migration would be performed in order to assess to what extent dissemination of bacteria to the thymus is dependent on those cells.

Clumps of infected macrophages and DCs are localized in the medulla and cortico-medullary region

Histological analysis of *M. avium* infected thymi reveals that *bacilli* are mainly localized at the medulla and cortico-medullary regions. In the first weeks of infection, histological analysis reveals few *bacilli* in the thymus at the cortico-medullary region; at later time points, large clumps of highly infected macrophage-like cells can be easily observed in the medulla and at the cortico-medullary region. In *M. tuberculosis* infected thymi, also at later time points, aggregates of infected cells are detected in the medulla and, to a lower extent, in the cortex and cortico-medullary region. It is interesting to note that Li *et al.* have shown in a parabiotic system that partner-derived DCs are mainly localized in the medulla and cortico-medullary regions of the thymus (37), which supports the possibility that DCs might enter the thymus, in this region, carrying mycobacteria from the periphery. Moreover, other cell types, such as bone marrow T cell precursors and peripheral T cells, also enter the thymus at the cortico-medullary region (40, 41) and medulla (41), respectively.

Immunofluorescence staining of infected-thymi reveals that most of the infected cells are $CD11c^{+}F4/80^{+}$ and, to a lower extent, $CD11c^{+}F4/80^{-}$. These phenotypes are consistent with the ones of foamy macrophages and DCs, respectively, described previously in the lungs of *M. tuberculosis* infected mice (42). However, it should be taken into account that depending on the tissue, the same markers can represent different cell subsets that are functionally distinct (42). Sköld and Behar have shown that upon *M. tuberculosis* infection,

monocytes are recruited to the lung and differentiate into five distinct myeloid subsets. Among those subsets, foamy macrophages represented the major cell type expressing iNOS in the lung whereas in the draining LN, cells with this same phenotype were not able to express iNOS and exhibit traits of mature resident DCs ($CD86^{high}CD83^{+}$) (42). Even though most of the infected cells in the thymus have, by conventional histology, a morphology consistent with the one of foamy macrophages (43), the classification of the infected cell-subsets based only on F4/80 and CD11c expression may be an oversimplification. Nonetheless, it is important to retain that only cells of hematopoietic origin are found infected; no infected epithelial cells are detected within the thymus. Despite this, a more detailed characterization of the infected cells in the thymus, during the course of infection, should be performed in order to further characterize the infected cells. Due to the necessity of using a wide array of markers for this purpose, this analysis should be performed by flow cytometry in mice infected with mycobacteria expressing a fluorescent marker (such as GFP). Markers as CD11c, CD11b, CD8, CD80, CD86, F4/80, iNOS and MHC class II should be assessed in order to characterize host's cells. Moreover, as described recently, Sirpα could also be a suitable marker in determining if infected DCs are of extrathymic origin ($Sirp\alpha^{+}CD11b^{+}CD8\alpha^{-}$) (36, 37).

In the murine model of mycobacterial infections the formation of granulomas occurs in most of the organs studied though the presence of caseous necrosis is not a common feature of these structures in mice. Interestingly, despite the large infiltrates of macrophage-like cells in the thymus, no signs of granulomatous organization are detected. On the contrary, the few human case reports on thymic infection with *M. tuberculosis* had in common the presence of granulomas with caseous necrosis (21-29). Whether the formation of granulomas is a common feature of thymic infection in humans or, on the contrary, it is also infrequent, is unknown. It is possible that the few human case reports on thymic infection result from the fact that in humans, like we observe in mice, thymic mycobacterial infection does not result, in most situations, in the formation of granulomas. To investigate this possibility, analysis of specimens acquired either *post-mortem* or from cardiac surgeries, from individuals with or without symptoms of mycobacterial infections should be performed. This examination would not only include histological analysis of the specimens but also culture of tissue homogenates and PCR analysis in order to identify mycobacteria-positive samples. This would allow, on one hand, to evaluate the frequency of mycobacterial infection of the thymus in humans and, on the other hand, the histological analysis of the thymi harbouring mycobacteria. In fact, we have initiated this work in the laboratory and detected one infected thymus, by PCR, out of 30 *post-mortem* thymic samples. It should however be referred that autopsy is

avoided in patients known to die from tuberculosis for the sake of health safety of the professionals. Likewise, it is not surprising that the number of positive samples is low. More samples will continue to be analyzed in the future.

Evidence for a delayed immune response against mycobacteria within the thymus

Mice infected intravenously with BCG are naturally able to control the bacterial load in the spleen, liver and lung (32, 44, 45). In these organs, an increase in the bacterial load is observed up until 2 weeks post infection; from this time point on, an evident decrease in the bacterial load is observed in the lung and liver. Interestingly, we observe that in the thymus bacteria are only detected by 4 weeks post infection; from this time point onwards, until the end of the experiment (12 weeks post infection), bacterial load in the thymus keeps rising. These results provide strong evidence that the control of the progression of the infection in the thymus is somehow distinct from the one in the other organs.

Infection with an intermediate virulence strain of *M. avium* results in a *plateau* in the bacterial growth curve of the spleen and liver no later than 4 weeks post infection. Unlike these organs, the bacterial load in the thymus increases for longer periods and the *plateau* in the bacterial growth curve is attained only around 16 weeks post infection. Of notice, preliminary data from our lab (not provided in this thesis), show that IFN-γ production in the thymus is only detected at 16 to 17 weeks post infection (assessed in cell culture supernatants and by mRNA levels of infected *versus* non-infected thymi). It has been previously shown that a strong immune response, characterized by an increase in the IFN-γ levels in cell culture supernatants of splenocytes stimulated with *M. avium* antigens (46) and in the spleen mRNA levels of IFN-γ and TNF (47-49), is detected in the spleen as early as 4 weeks post infection. Thus, our preliminary results suggest that the immune response in the thymus is delayed in comparison with that occurring in the spleen.

A more detailed dissection of the cytokine profile in the thymus upon infection should be performed. mRNA levels as well as protein levels of the cytokines that are usually involved in the immune response to mycobacterial infections (such as IFN-γ, TNF, IL-2, IL10 and IL12), should be assessed in the spleen and thymus of *M. avium*-infected mice, and in age-matched controls, over a set of time-points during the course of the infection. The functional ability of thymocytes and splenocytes to produce cytokines upon *in vitro* stimulation with mycobacterial antigens should also be measured in these animals. This analysis is essential to understand the kinetics of the immune response in the thymus.

Under steady-state conditions, the main role of the cytokines produced in the thymus is to induce constitutive processes such as migration and development of thymocytes and regulation of cell number (50). In fact, the altered expression of cytokines in the thymus such as IFN-γ, TNF, IL-2 and IL-10 (which are also cytokines normally produced upon mycobacterial infections) can affect the normal T cell differentiation in the thymus (50). It has been demonstrated that, for example, IFN-γ production influences thymocyte adhesion to TECs and thymocyte exit from nurse cells. Moreover, IFN-γ can also modulate thymocyte proliferation via stimulation of IL-6 secretion by TECs (50). For these reasons, understanding the profile of cytokine production in infected thymi will also be useful to evaluate the potential impact of such cytokines on the normal function of the thymus.

It would also be interesting to explore the cell players involved in the thymic immune response. Re-entry of peripheral T cells into the thymus has been previously described (41, 51, 52). Once within the thymus, it has been suggested that peripheral T cells can play an active role in several processes such as positive and negative selection of thymocytes (53, 54) and differentiation and organization of a normal thymic stroma (55). The influx of peripheral T cells in the adult thymus is restricted to activated T cells (41, 51, 52). Our preliminary data (not provided in this thesis) shows that peripheral T cells do re-enter the thymus during *M. avium* infection. It was observed that when TCRα KO mice (infected for 4 weeks with *M. avium*) receive spleen cells from an infected congenic pair (Ly5.1 mice also infected for 4 weeks), donor cells can be detected in the thymi of TCRα KO mice at 2 weeks post transfer. Considering this, it would be interesting to assess whether peripheral T cells participate in the immune response to thymic infection. This could be easily assessed in RAG2-GFP mice infected with *M. avium.* These mice express GFP under the control of the RAG2 promoter (56). During thymocyte differentiation RAG2 is expressed throughout the rearrangement of the TCR; even though RAG2 is down regulated after thymic selection, cells are kept as GFP^+ for about 2 weeks. This system enables the discrimination, within the thymus, of newly differentiated CD4 SP or CD8 SP cells (GFP^+) from the peripheral mature T cells that re-enter the thymus from the periphery (GFP^-) (57, 58). By performing intracellular staining for specific cytokines, we could identify by flow cytometry whether peripheral T cells (GFP^-) or newly differentiated T cells (GFP^+) are involved in the control of the bacterial load in the thymus.

Thymic infection with *M. avium* does not reduce *de novo* T cell generation

Most reports on thymic infection by pathogens other than mycobacteria, describe cortical atrophy, with loss of the cortico-medullary boundary, as one of the most common consequences of those infections (8, 9, 11, 14, 16-20). This atrophy was related to a massive decrease of the DP thymocytes mainly by apoptosis. Moreover, cortical atrophy could also be induced by bacterial constituents such as mycobacterial cord factor and LPS (59, 60). In models where infection was performed with two different strains of the same pathogen [*F. tularensis* (20), measles virus (14) or with SIV (11)] premature thymic atrophy was only observed for one of the strains, indicating that thymic atrophy is dependent on specific characteristics of the invading pathogen. Additionally, atrophy could also be reverted with the clearance of the infectious agent (61, 62). The precise mechanisms involved in thymic atrophy are not completely understood and may vary with the infecting agent.

In our model of *M. avium* infection, despite the accumulation of highly infected cells in the medulla and at the cortico-medullary region, the cortex can still be easily distinguished from the medulla; no visual depletion of cortical thymocytes can be detected. Nevertheless, this analysis was taken further by assessing thymic cellularity as well as the percentages of the main thymic populations (assessed by CD4 and CD8 expression: DN, DP, CD4 SP and CD8 SP) in infected animals *versus* age-matched non-infected controls. No major differences are observed in these populations except for a minor decrease in the percentage of DP, late in infection, alongside with a small increase in the percentages of the SP populations. The decrease in the percentage of DP thymocytes is not as severe as the one observed in other models of infection (8, 9, 11, 14, 16-18, 20) and may be due to an increase in the percentages of CD4 SP and CD8 SP populations associated to an influx of mature peripheral T cells (41, 51, 52) due to the infection, as we observe no reduction of the total thymic cellularity. Our results are in agreement, and extend to longer time points the ones published by Florido *et al.* (49). In this report no premature thymic atrophy was detected during the first 8 weeks of infection with an intermediate-virulence strain of *M. avium* (strain 2447, the same used throughout our work) (49). It is interesting to note however that when Florido *et al.* used a high-virulence strain of *M. avium* (strain 25291) (49), that also infects the thymus (32), a strong decrease in the thymic cellularity was observed as well as a diminished number of DP cells in infected thymi (49).

One of the main consequences of premature thymic atrophy is the decrease in thymic output. Despite all the caveats associated to the indirect quantification of thymic output by analysis of TRECs (63), it was shown that both HIV-infected individuals as well as non-human primates infected with SIV, exhibit lower TREC levels in the peripheral blood, later

in infection, in comparison to non-infected age-matched controls (64-66). This decrease has been associated not only to an increased proliferation of peripheral T cells (assessed by the increased percentage of Ki67$^+$ cells and augmented incorporation of BrdU) as well as to decreased thymic output associated to thymic pathology (65, 66). In contrast, it has been observed in patients undergoing HAART, a marked increase in TREC amount and of thymic volume, which were indicators of restored thymic function (67, 68). Herein we have also assessed the ability of *M. avium*-infected thymi to support T cell differentiation by quantification of TRECs. Due to the high proliferation rates observed in the spleen of infected mice (that could lead to an underestimation of thymic output in infected mice), we have quantified TRECs in thymi of infected *versus* non-infected age-matched controls. No differences are observed in the TREC amount up to 30 weeks post infection, which is a good indicator that infected thymi are equally able to support T cell differentiation in comparison to non-infected thymi. Another indication that infected thymi are equally able to produce T cells as non-infected thymi is the observation that, when transplanted into the kidney capsule of nude mice, the levels of peripheral T cell reconstitution 8 to 9 weeks post-transplant is similar in both groups.

In summary, herein we show that, in comparison to age-matched controls, infection of the thymus with an intermediate-virulence strain of *M. avium*: i) does not lead to premature thymic atrophy; ii) is not responsible for major changes on the thymocyte populations; iii) does not impact on TREC amount in the thymus; iv) leads to the production of T cells that equally replenish the periphery, as the ones produced by non-infected thymi, when transplanted to nude mice. Altogether, these observations are good indicators that infected thymi have similar capacity to produce T cells as non-infected thymi.

M. avium within the thymus leads to the differentiation of T cells tolerant to *M. avium*-derived antigens

Central tolerance mechanisms are essential to generate a pool of peripheral T cells that is mostly self-MHC restricted and unreactive to self-peptides. Tolerance to self-peptides results mainly from clonal deletion of the potentially autoreactive thymocytes. Other mechanisms of central tolerance can also occur such as induction of anergy and receptor editing. Nevertheless, central tolerance mechanisms are not full-proof and some potentially autoreactive T cells may escape and colonize the periphery. In this situation, peripheral tolerance mechanisms play a role in avoiding or limiting the damage of those self-reactive mature T cells. Though clonal deletion and anergy also play a role in the

induction of peripheral tolerance, the main players are the regulatory T cells that suppress the activity of self-reactive T cells (69-71).

Tolerance mechanisms are highly dependent on the interaction of the T cell with the cognate antigen. For central tolerance mechanisms, two sources of antigens in the thymus have been described, both leading to the elimination of autoreactive T cells: i) expression of TSAs by mTECs that can be presented by themselves (72, 73) or cross-presented by DCs (74); ii) influx of DCs loaded with peripheral antigens (35). The entrance of antigen-loaded DCs into the thymus offers the ability to present antigens that are not expressed by thymic cells (38, 75). Our results, using nude mice transplanted with thymic lobes, show that the presence of *M. avium* within the thymus leads to the production of T cells that have reduced ability to protect to a subsequent infection, with the same pathogen, in comparison to T cells that differentiated within non-infected thymi. Mice reconstituted with T cells that differentiated within infected thymi have an increased bacterial load in the spleen and liver in comparison to the ones with T cells that differentiated in non-infected thymi. Additionally, when splenocytes from these two groups of mice were stimulated *in vitro* with *M. avium*-derived antigenic fractions, T cells differentiated within infected thymi failed to produce IFN-γ in response to most of the fractions tested. This impaired ability to respond *in vitro* to stimuli was specific for mycobacterial antigens as splenocytes from mice transplanted with infected or non-infected thymi, and immunized with OVA, exhibit similar proportions of IFN-γ producing T cells. Several mechanisms may underlie this induced tolerance such as anergy, clonal deletion of specific T cells and/or development of regulatory T cells that peripherally suppress the activity of effector T cells; the involvement of each of these mechanisms will be discussed next.

Anergy means that T cells have lost their ability to proliferate and have a decreased production of cytokines in response to stimuli (76, 77). In our experiments, two main observations may provide evidence that anergy is not the tolerogenic mechanism underlying mycobacterial tolerance. First, splenocytes from OVA-immunized nude mice transplanted with infected or non-infected thymi, when stimulated *in vitro* with the immunogen, have similar percentage of cells producing IFN-γ. This observation is a strong indication that T cells differentiated within infected-thymi are specifically unable to respond to mycobacterial antigens, and not to an unrelated antigen. Second, T cells that differentiated within infected or non-infected thymi are equally able to proliferate once they are transferred to a new empty host. This is a strong indicator that these cells have no impaired proliferation ability. Altogether, these observations show that the T cell population generated within infected thymi is not an anergic population; however, we

cannot exclude the hypothesis that, among the T cells differentiated within infected thymi, T cells specific for *M. avium* antigens are anergic. This is a subject that should be further explored. For this, the use of tetramers specific for mycobacterial peptides would be extremely useful; this tool would enable the identification and subsequent characterization of the T cells specific for a mycobacterial peptide. Not only functional studies could be performed with these cells as the molecular analysis of molecules downstream the TCR signalling pathway could be assessed [such as the phosphorilation level of Zap-70/Syc or of LAT (linker of activated T cells) which is impaired in anergic states].

The localization of the *bacilli* at the cortico-medullary region and medulla favours the hypothesis that mycobacteria-specific T cells are deleted since it is well established that the medulla is the specialized site for clonal deletion (71). DCs are highly efficient in supporting clonal deletion (78) and they are, together with macrophages, the cells harbouring mycobacteria within the thymus. It could be argued that, since few DCs are infected within the thymus, it would be unlikely for clonal deletion, of thymocytes reactive to mycobacterial antigens, to occur supported by DCs. Nevertheless, it has been proposed that the high motility of thymocytes as well as their long-term permanence in the thymus facilitate the interaction of thymocytes with thymic stroma thus favouring clonal deletion (69). Even though it is now accepted that thymocytes spend 4 to 5 days in the medulla (58), as opposed to the 10 days proposed earlier (79, 80), this time-period is sufficient for clonal deletion of potentially auto-reactive thymocytes to occur (81). In fact, it was shown that a single transfer of antigen-loaded DCs into WT mice was sufficient to support clonal deletion of thymocytes reactive to that specific antigen (35). This report stresses the importance of DCs as antigen presenters relatively to their numeric representation in the thymus.

Although our results suggest clonal deletion as the main mechanism responsible for tolerance to mycobacteria in infected thymi, it will be necessary to show that this is in fact the case. The simplest way to assess if *M. avium*-specific thymocytes are being deleted, upon encountering their cognate antigen within the thymus, is by analyzing populations of T cells expressing specific transgene-encoded TCRs that recognize antigens expressed by mycobacteria. Two TCR-transgenic mouse models are currently available: i) the P25 mouse line, whose TCRs specifically recognize a peptide from Ag85 expressed both by *M. avium* and *M. tuberculosis* (82); ii) ESAT-6 mouse line in which TCRs specifically recognize the ESAT-6_{1-20} peptide secreted by *M. tuberculosis* (83). Both transgenic lines have T cells whose TCR recognizes the peptide in the context of MHC class II I-A^b molecule. If negative selection contributes to the tolerance to mycobacteria, one expects

to see, by flow cytometry, a reduction on the most mature thymocytes expressing the transgenic TCRs (CD4 SP) while infection of this organ progresses.

Another possible strategy would be infecting transgenic mice expressing TCRs that recognize OVA with OVA-expressing mycobacteria . To do so, three mouse lines would be useful: the OT-I (84), OT-II (85) and the *Limited* (86) mouse lines. Both OT-I and OT-II mouse lines express TCRs that recognize an OVA-peptide either in the context of MHC class I or MHC class II, respectively. *Limited* mice, is a mouse line with transgenic TCRs that differ from each other exclusively on the CDR3 of the TCRα chain; one of the dominant TCR sequences is the reconstitution of the original OT-I TCR. Using the OT-I and OT-II mouse line, clonal deletion could be easily assessed by FACs analysis: if clonal selection is taking place, a reduction in the proportion of mature SP thymocytes (CD4 SP or CD8 SP depending on whether OT-II or OT-I mice are used, respectively) is expected upon infection with OVA-expressing mycobacteria. As a control, WT mycobacteria would be used. To address clonal deletion using *Limited* mice it would be necessary to analyse the DNA sequences of the mature CD8 SP thymocytes; if clonal deletion is occurring a reduction in the proportion of thymocytes expressing the original OT-I TCR would be expected.

It is also possible that regulatory T cells generated in infected thymi exert a suppressive effect on mycobacteria-specific T cells. Several reports have shown that naturally arising regulatory T cells originate in the thymus although the cell subsets that mediate the selection of these cells is still a matter of debate (87). In a TCR-transgenic model, Jordan *et al.* have shown that $CD4^+$ T cells specific for the cognate antigen were selected into the $CD25^+$ lineage when the agonist ligand was expressed under the control of an ubiquitous promoter (88). In another system, transplantation of thymi from antigen-transgenic mice into antigen-deficient hosts was sufficient to direct the selection of a large fraction of specific $CD4^+$ T cells into the regulatory T cells lineage (89, 90). cTECs (91-94), mTECs (95-98) and/or DCs (36, 98, 99) have been implicated in the generation of regulatory T cells. A recent report has described DCs of extrathymic origin ($CD8^{low}Sirp\alpha^+$) as contributors to the induction of regulatory T cells (36). This was shown when irradiated WT mice were reconstituted with bone marrow from MHC class II KO mice; these chimeras (which had a hematopoietic compartment impaired in antigen presentation) had a decreased number of thymic regulatory T cells in comparison to chimeras that had received WT bone marrow (36). Moreover, it was also shown that $CD8^{low}Sirp\alpha^+$ DCs (which are DCs with extrathymic origin) have increased capacity to induce regulatory T cells, *in vitro*, in comparison to the other DC subsets (36).

Even though thymic DCs are a target for mycobacteria within the thymus, which could favour the generation of regulatory T cells, similar numbers of splenic $CD4^+CD25^+Foxp3^+$ cells are generated by infected or non-infected thymi. This is a strong indicator that, if the tolerogenic effect observed is related to regulatory T cells this would be a matter of specificity, and not of number of these cells. Histological data does not support this hypothesis. Liver lesions of mice with T cells that differentiate within infected thymi are extremely similar with lesions from nude mice. This suggests the lack of specific T cells able to induce the formation of more organized granulomas containing activated macrophages (assessed by the expression of iNOS). If the lack of T cell response was due to the suppressive effect of the regulatory T cells, lesions similar to WT mice would be expected since regulatory T cells were described to exert their function not only in the LN, but also in lung granulomas of *M. tuberculosis* infected WT mice (100).

One way to assess the relevance of specific regulatory T cells would be by performing a transplant system, similar to the one we have used previously in this work, but using bone marrow from Foxp3-GFP mice as a source of T cell precursors. In Foxp3-GFP mice, a nuclear chimeric Foxp3-GFP fusion protein is expressed on regulatory T cells (96) enabling the distinction of conventional $CD4^+$ T cells ($CD4^+GFP^-$) from regulatory T cells ($CD4^+GFP^+$). To do this experiment, recipient mice would be TCRα KO mice previously thymectomized and irradiated; infected or non-infected thymic lobes (from TCRα KO) would be transplanted under the kidney capsule of these mice. In this situation, the transferred bone marrow T cell precursors would differentiate in the transplanted thymi. 8 to 10 weeks later (the time required for the replenishment of the periphery with T cells) total $CD4^+$ T cells and $CD4^+$ T cells depleted from regulatory T cells (conventional $CD4^+$ T cells) would be sorted and stimulated *in vitro* with mycobacterial specific antigens. If regulatory T cells play an important role in tolerance induced to mycobacteria, the IFN-γ production by conventional T cells (depleted from regulatory T cells) should be similar for T cells differentiated in infected or non-infected thymi. Moreover, isolating conventional $CD4^+$ T cells and regulatory T cells from mice with T cells differentiated within infected or non-infected thymi would also enable *in vitro* studies of the suppressive activity of regulatory T cells from each group or mice.

Altogether, the proposed experiments should help clarify the mechanisms underlying the decreased ability of T cells that differentiate within infected thymi to mount an efficient immune response to *M. avium* infection.

Newly differentiated T cells and the progression of chronic *M. avium* infection

An increasing body of evidence has shown that, despite the normal age-related thymic atrophy, the thymus remains functional during adulthood, maintaining its ability to produce new T cells (101-104). This ability to produce T cells during adulthood becomes particularly evident in situations of strong lymphopenia. Several reports have assessed the role of thymic output in the maintenance of the peripheral T cell pool. In humans, only a modest decrease in the amount of blood T cells was observed, in comparison to age-matched controls, 10 years after thymectomy performed during the first year of life (105-109). However, in the mouse model, Dai *et al.* and Di Rosa *et al.* have observed a marked decrease in the numbers of spleen $CD4^+$ T cells at 10 (110) and 26 (111) weeks post thymectomy, respectively. More recently, Bourgeois *et al.* have confirmed these observation in a model of genetic thymectomy (by conditional ablation of RAG2 gene expression); a decrease of about 50% in the amount of $CD4^+$ T cells 8 weeks after total ablation of thymic export was observed (112). Of notice, the naive T cell pool was highly affected by the cessation of thymic T cell export whereas memory and regulatory T cells were maintained in constant numbers (112). Despite the continuous export of new T cells by the thymus during adulthood and their role in maintaining the peripheral T cell pool, few reports have explored the role of these cells in the course of mycobacterial persistent infections. Winslow *et al.* have shown that thymectomy prior to *M. tuberculosis* infection, does not induce major changes on the percentage of lung ESAT-6_{1-20} specific $CD4^+$ T cells of thymectomized mice at 50 days post infection (113). However, we observe that *M. avium* infection of thymectomized mice leads to a marked decrease in the percentage of spleen $CD4^+$ T cells from 4 weeks post infection on, in comparison to sham-thymectomized mice. It should be noticed that Winslow *et al.* assessed lung $CD4^+$ T cells that specifically recognize the ESAT-6_{1-20} peptide (113) whereas we analysed total spleen $CD4^+$ T cells; not only different organs but also different cell populations are addressed which may underlie the different results obtained.

When the bacterial loads were assessed in thymectomized *M. tuberculosis* infected mice, Winslow *et al.* showed that thymectomy does not induce major changes in the *M. tuberculosis* bacterial load, suggesting that newly generated T cells are not relevant for the outcome of chronic infection (113). In the context of *M. avium* infection, it was shown that upon infection of thymectomized mice, with an intermediate-virulence strain of the bacteria, no differences were observed in the bacterial load in the spleen and liver (48, 49) or in the IFN-γ and TNF mRNA levels in the spleen up until 60 days upon infection (48). Our results extend these observations up until 31 weeks post infection. However, it should be noticed that when a high-virulence strain of *M. avium*, which induces peripheral

lymphopenia was used, Florido *et al.* showed that thymectomyzed mice are more susceptible to infection in comparison to non-thymectomized animals (114). This observation indicates that upon depletion of the peripheral T cell pool, thymic output gains a relevant role in the establishment of the immune response to mycobacterial infections.

The observation that thymectomy does not alter the course of mycobacterial infections, with strains that do not induce peripheral lymphopenia, results most probably from the fact that T cells present in the periphery, at the moment of thymectomy, are sufficient to mount and maintain this response. However, we know now that *de novo* generated T cells from infected thymi are tolerant to mycobacteria. One should then consider the possibility that this situation might also contribute to the lack of a role of the newly generated T cells in the outcome of the infection.

To clarify whether newly differentiated T cells, that are not tolerant to mycobacteria, might play a role in the outcome of *M. avium* chronic infection it would be necessary to generate a system where infected mice would receive T cells from non-infected thymi. To achieve this, naive T cells would be periodically transferred into thymectomized and infected mice. The bacterial loads of these mice would be compared, at different time points, to the bacterial loads of thymectomized infected mice not receiving cells. This experiment could be further improved by transferring a purified population of recent thymic emigrants instead of spleen T cells from naive mice. This population could be obtained from RAG2-GFP mice. As referred previously, these mice express GFP under the control of the RAG2 promoter (56) and, for this reason, T cells are GFP^+ for about 2 weeks upon down-regulation of the RAG2 gene. This allows the discrimination in the periphery of recent thymic emigrants (GFP^+) from T cells that were already in the periphery (GFP^-) (58).

Potential consequences of the generation of tolerant T cells by mycobacteria infected thymi

As referred previously, in the setting of severe T cell depletion, due to HIV infection, cytoreductive transplantation or chemotherapy regimens, thymic output regains a relevant role in the re-establishment of the immune system (115). Hakim *et al.* demonstrated that after autologous transplant, renewed thymopoiesis is critical for the restoration of peripheral $CD4^+$ T cells. This recovery encompasses the recovery of normal $CD4^+$ T cell numbers with a broad repertoire of TCR diversity among these memory cells (115). Moreover, for HIV^+ patients following HAART, those with larger increases in thymic volume have shown larger increases in $CD4^+$ T cell numbers over the course of therapy (116-118). These results support the importance of the thymus not only until early

adulthood but also for the elder, especially in cases of lymphopenia. Considering the possibility that, in some cases, the thymus may be infected with mycobacteria in humans, this would have deleterious effects on the ability to control ongoing or subsequent mycobacterial infections, as we observe in our animal model.

Despite all the potential consequences described above, one might also relate the infection of the thymus by BCG with the decreased protection induced by this vaccine. BCG is the only vaccine available to prevent tuberculosis; however the efficacy provided is highly variable and, in populations where it is known to induce protection, it is only for the first 10 to 20 years of age (119). The mechanisms underlying this loss of protection are highly controversial. In this work, we have shown that BCG, when administered intravenously, can reach the thymus and persist in this organ, even when the bacterial load in spleen and liver are already decreasing (as discussed earlier). Moreover, we have also shown that *M. avium* within the thymus can lead to the generation of T cells unable to protect in a subsequent infection with the same pathogen. Taking all of this into account, one may speculate that, upon vaccination, BCG could disseminate and persist in the thymus leading to the production of a T cell population unresponsive to mycobacterial antigens. Nevertheless, it should be said that in our model of BCG infection we have inoculated the bacteria intravenously; in the case of BCG vaccination this is performed subcutaneously. We have attempted this route of inoculation in mice and preliminary results (not provided in this work) reveal a high variability in the extent of thymi infection; not all mice get their thymi infected and the ones that are infected exhibit high variability in the bacterial load. Despite the great variability, this observation is a good indicator that, even upon subcutaneous inoculation of BCG, *bacilli* might reach the thymus.

Mycobacteria within the thymus are more difficult to eliminate upon antibiotic treatment

The efficacy of antibiotic treatments is known to depend on variables such as dose, schedule of administration, formulation and target organs (120). Herein we show that antibiotics (clarithromycin, ethambutol, and rifampicin) administered in the drinking water to *M. avium*-infected mice are not as efficient in eliminating *bacilli* in the thymus as they are in other organs. Antibiotics administered in the drinking water have a bactericidal effect in the spleen, liver and lung though in the thymus that effect is merely bacteriostatic. Of notice, when similar doses of antibiotics are administered by gavage, a bactericidal effect is observed in all organs assessed, including the thymus. Nevertheless, the reduction of the bacterial load in the thymus is not as pronounced as in the other organs.

This observation was further confirmed by the calculation of the D-values; more than twice the time is required to reduce by 1log CFU the bacterial load in the thymus than in the other organs when antibiotics are given by gavage. Despite the different effect of the antibiotics depending on the mode of administration, our results clearly indicate that the efficacy of antibiotic treatment is lower for *bacilli* within thymus. One reason that could account for this decreased effect is the presence of the BTB that could lead to a reduced entry of the antibiotics into the thymus, namely clarithromycin that is one of the largest molecules in the antibiotic cocktail, and the most efficient in controlling *M. avium* growth (121-124). However, when we omit clarithromycin from the antibiotic cocktail, not even a bacteriostatic effect is attained in the thymus, suggesting that clarithromycin enters the thymus and is essential to induce a bacteriostatic effect. Moreover, these results provide evidence that only the combination of the three drugs is able to induce some effect on the bacteria in the thymus, even if that effect is merely bacteriostatic.

The decreased effect of antibiotic treatment on the control of the bacterial load in the thymus, in comparison to bacteria in other organs, may be related to the different/delayed immune response established in the thymus. Several reports showed that the efficacy of the anti-mycobacterial drugs can be improved with the modulation of the immune response, either by blocking the IL-10-receptor (125, 126), by the administration of recombinant IL-12 (127) or TNF (128). A synergistic effect of IFN-γ administration and antibiotic treatment was also observed in patients with MAC infections (129). For this reason, it would be interesting to assess the efficacy of antibiotic treatment in the thymus of IL-10 KO *versus* WT mice. Moreover, it would also be interesting to study the antibiotic effect in the thymus upon induced over-expression, exclusively in the thymus, of specific cytokines such as IL-12 or IFN-γ. This could be achieved by intrathymic injection of non-replicating adenoviral vectors expressing the IL-12p35 and IL-12p40 subunits (130, 131) or IFN-γ (132).

The possibility that bacteria develop in the thymus a different metabolic state that would be responsible for their increased resistance to the antibiotic treatment should also be considered. This is in agreement with a report showing that, in culture, *M. avium* can enter a reversible state of metabolic dormancy. In this situation, increased resistance to antibiotic treatment is observed (133). However, the existence of a metabolically dormant state for *M. avium* has been poorly explored and no evidence exists that this can happen for *M. avium* within host's cells.

Recently, it was demonstrated that both *M. avium* and *M. tuberculosis*, but not *Mycobacterium smegmatis* (a non-pathogenic mycobacteria), are able to induce the differentiation of human monocytes into foamy macrophages. The authors have

suggested that oxygenated mycolic acids, which are both present in *M. avium* and *M. tuberculosis* but not in *M. smegmatis,* would be responsible for this differentiation process. They have further showed that, within foamy macrophages, *M. tuberculosis* enters into a dormant non-replicative state shown not only by the decreased bacterial proliferation rate but also by the increased up-regulation of dormancy genes. *M. tuberculosis*-dormant *bacilli* exhibit increased resistance to antibiotic treatment (134). Unfortunately, they did not explore whether *M. avium* could also enter this state of dormant *bacilli* within foamy macrophages. Nonetheless, these observations were quite interesting since we are able to identify *M. avium*-infected foamy macrophages within the thymus; this might give an indication that *M. avium* in the thymus might undergo metabolic changes and, for this reason, become less susceptible to antibiotic treatment. For all this, it is pivotal to compare the transcription profile of *bacilli* within the thymus *versus bacilli* in other organs such as spleen and/or liver over the time-course of infection.

The effect of antibiotic treatment during *M. tuberculosis* infection was not studied in the context of this thesis. If antibiotic treatment has also a decreased efficacy on *M. tuberculosis* in the thymus, this could place the thymus as a potential site responsible for tuberculosis reactivation and/or latency. In the thymus, *bacilli* would not only be "protected" from antibiotic treatment but would also be in an organ where the immune response is, putatively, slower/weaker in comparison to other organs (as discussed earlier), which would be an ideal condition for the establishment of latent infections. For this reason, it is essential to explore the antibiotic efficacy on *M. tuberculosis* within the thymus as well as to investigate this organ as a putative site of *M. tuberculosis* latency.

The Cornell model (135, 136), as well as the subsequent variations of this model (137), enable the study of latent tuberculosis reactivation. In these models, mice are infected with *M. tuberculosis* and treated with antibiotics until no viable *bacilli* are detected by organ culture (usually the lung and spleen are the organs assessed); reactivation of the latent infection occurs either spontaneously or following immunosuppression (135-137). When using this model in thymectomized and sham-thymectomized mice one could compare the time required in each of the groups for reactivation. If thymectomized mice were to require longer time to reactivate tuberculosis, in comparison to sham-thymectomized mice, this would be a good indicator that the thymus is a niche for latent *bacilli*. Nevertheless, in this situation, it is hypothesized that *bacilli* can leave the thymus and establish an infection in the periphery, which should be first investigated.

Finally, the persistence of the *bacilli* within the thymus could also lead to deleterious consequences on the modulation of the immune response. As discussed earlier, *bacilli* within the thymus are responsible for the generation of T cells unresponsive to

mycobacterial antigens. If *bacilli* persist within the thymus even after antibiotic treatment, bacteria would continuously be present during the differentiation of T cells leading to the continuous production of *de novo* generated T cells unable to respond to mycobacteria.

5.2. CONCLUDING REMARKS

In this work we show that during mycobacterial infections *bacilli* colonize the thymus leading to the generation of T cell that are tolerant specifically to mycobacterial antigens. Moreover, we show that antibiotic treatment has a decreased effect on the bacteria in the thymus in comparison to *bacilli* within other organs. This difficulty to eliminate bacteria in the thymus may anticipate the persistence of mycobacteria in this organ and lead to the production of T cells with impaired capacity to respond to mycobacterial infection. Our observations may be especially relevant in the reconstitution of the peripheral T cell repertoire after lymphopenic situations, namely in the case of HIV^+ individuals on HAART which are co-infected with mycobacteria. Altogether, these data stress the need to consider the thymus whenever new preventive and therapeutic strategies are developed. Among the several important questions that remained open in this dissertation, one must be highlighted: are the few case reports published so far on mycobacterial infection of the thymus in humans just the tip of an iceberg?

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