Bruno Miguel Cerqueira Rodrigues

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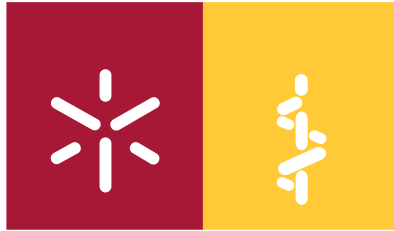
Junho de 2011

Universidade do Minho

Escola de Ciências da Saúde

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Dissertação de Mestrado
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Trabalho efectuado sob a orientação da
Doutora Cláudia Rubina Freitas Pereira de Nóbrega
e co-orientação da
Doutora Maria Margarida Teles Vasconcelos Correia Neves

Junho de 2011

Ao meu Pai, à Monica e à Carla

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Contact with mycobacteria is common worldwide. Attention is mostly directed to the massive number of infections with *Mycobacterium tuberculosis* (about 1/3rd of the world's population) and *Mycobacterium avium* in co-infected HIV patients and other immunocompromised individuals.

Our laboratory has recently shown that, in the mouse model, mycobacteria are able to infect the thymus, the organ responsible for T lymphocyte differentiation, rendering newly differentiated T cells tolerant in responding to mycobacterial antigens.

The bacterial load in the thymus slowly progresses, reaching a stagnation at more advanced periods of infection (16 weeks post-infection), while in the spleen it occurs after 4 weeks. In the spleen, this stagnation is clearly associated to the appearance of IFN-γ secreting T cells, and so we suspected the involvement of these cells in the thymus as well.

Indeed, coinciding with the stabilization of the bacterial load in the thymus is an increased expression of IFN-γ at 16 weeks post-infection (wpi), in infected mice, followed by an increase in iNOS expression, a marker of macrophagic activation, at 24wpi.

Knowing the immature phenotype of newly generated T cells in the adult mice, we suspected the involvement of mature T cells, which are re-circulating from peripheral organs back to the thymus, in the thymic mycobacterial infection. Using the transgenic RAG-GFP mice, that allow the discrimination of newly generated T cells (GFP·) from re-circulating T cells (GFP), we assessed the number, specificity and IFN-γ secreting ability of both these populations in the thymus. As previously described and further confirmed by our group, re-circulating T cells have a phenotype consistent with high expression levels of CD44 and low of CD24, allowing us to also extend this analysis to wild type mice.

Increased levels of the Th1 recruiting chemokines IP-10, MIG and MIP-1β was detected in the thymus from 16 weeks of infection in comparison to non-infected mice. Although the number of re-circulating cells was not increased by infection, this pool was enriched with specific mycobacterial T cells. We were able to detect an increased number of mycobacterial antigen (Ag)85-specific T cells within the pool of re-circulating cells after 16 weeks of infection, in wild type mice, and 20 weeks, in RAG-GFP mice.

Moreover, when transferring re-circulating T cells from RAG-GFP mice, with 20 weeks of infection, into infected mice with no $\alpha\beta$T cells (TCR$\alpha^{-/-}$), these cells were more capable of producing IFN-γ when stimulated specifically with Ag85 but not with PMA+ION. Furthermore, these cells also have a tendency to be more enriched with Ag85-specific T cells.

The data from this work presents evidence of an ongoing immune response in the thymus, which occurs at later time points and with a distinct activation profile from that in the spleen. The thymus recruits mycobacterial T cells from the periphery, which appear to be major producers of IFN-γ.

Contacto com micobactérias é comum mundialmente. A atenção é normalmente direccionada para o vasto número de infecções com *Mycobacterium tuberculosis* (estimado a ser 1/3 da população mundial) e com *Mycobacterium avium* em pacientes co-infectados com HIV e outros indivíduos imunocomprometidos.

O nosso laboratório mostrou que, no modelo do ratinho, as micobactérias são capazes de infectar o timo, o órgão responsável pela diferenciação de linfócitos T, gerando células recém diferenciadas que são tolerantes a responder a antigénios micobacterianos

A carga bacteriana no timo progride lentamente, estagnando em períodos mais avançados da infecção (16 semanas pós-infecção), enquanto que no baço esta ocorre após 4 semanas. No baço esta estagnação está claramente associado ao aparecimento de células T capazes de segregar IFN-γ, e sendo assim suspeitamos o envolvimento destas mesmas células no timo.

Deveras, coincidente com a estabilização da carga bacteriana no timo está um aumento da expressão de IFN-γ às 16 semanas pós infecção, em ratinhos infectados, seguido por um aumento da expressão de iNOS, um marcador de activação macrofágica, às 24 semanas de infecção.

Sabendo do fenótipo imaturo das células T recém diferenciadas em ratinhos adultos, suspeitamos do envolvimento de células T maturas, que estão e re-circular de órgãos periféricos de novo para o timo, na infecção micobacteriana do timo. Usando ratinhos transgénicos RAG-GFP, que possibilitam a distinção entre células T recém diferenciadas (GFP⁺) e células T que estão e re-circular (GFP), fomos estudar o número, a especificidade e a capacidade de produzir IFN-γ de ambas estas populações celulares no timo. Como previamente descrito e posteriormente confirmado pelo nosso grupo, células T em re-circulação possuem um fenótipo consistente com elevados níveis de expressão de CD44 e baixos de CD24, possibilitando a extensão desta análise a animais não mutantes.

Níveis elevados de quimiocinas recrutadoras de Th1 IP-10, MIG e MIP-1β foram detectadas no timo a parir das 16 semanas de infecção. Apesar do número de células em re-circulação não estar alterado pela infecção, esta população estava enriquecida com células T específicas para micobactérias. Também detectamos um aumento de células T específicas para o antigénio (Ag)85 micobacteriano dentro da população de células a re-circular após 16 semanas de infecção, em animais não mutantes, e 20 semanas, em animais RAG-GFP.

Transferindo células T em re-circulação de animais RAG-GFP, com 20 semanas de infecção, para animais sem células αβT (TCRα⁻⁄⁻), estas eram mais capazes de produzir IFN-γ quando estimulados com Ag85 mas não com PMA+ION. Além disso, estas células aparentam ter uma tendência para serem mais enriquecidas com células T específicas para Ag85.

Os dados destes trabalho evidenciam uma resposta imune no timo, que ocorre mais tardiamente e com um perfil de activação distinto daquele que ocorre no baço. O timo recruta células T específicas para micobactérias da periferia, que aparentam ser produtoras de IFN-γ.

Table of Contents

αβT cell – alpha-beta T cell

Ag85 – antigen 85

APC – antigen presenting cell

BCG – Mycobacterium bovis Bacillus Calmette-Guerin

BM – bone marrow

CCR7 – CC chemokine receptor type 77

CDR – complementarity determining regions

CFU – colony forming unit

CMJ – cortico-medullary junction

ConA – concanavalin A

cTEC – cortical thymic epithelial cell

CXCR3 – CXC chemokine receptor 3

DC – dendritic cell

DN – double negative

DP– double positive

ELC – EBI1-ligand chemokine

γ∂T cell – gamma-delta T cell

GFP – green fluorescent protein

HPRT – hypoxanthine-guanine phosphoribosyltransferase

HIV – human immunodeficiency virus

Ig – immunoglobulin

IL – interleukin

IFN-γ – interferon-gamma

iNOS – inducible nitric oxide synthase

ION – ionomycin

IP-10 – interferon-inducible protein-10

I-TAC – interferon-inducible T cell alpha chemoattractant

i.v. – intravenous

KO – knockout

LAM – lipoarabinomannan

LPS – lipopolysaccharide

Mφ – macrophage

MAC – *Mycobacterium avium* complex

MIG – monokine induced by gamma interferon

MIP-1α – macrophage inflammatory protein-1alpha

MIP-1β – macrophage inflammatory protein-1beta

MHC – major histocompatibility complex

mTEC – medullary thymic epithelial cell

NO – nitric oxide

OVA – ovalbumin

PAMP – pathogen associated molecular patterns

PMA – phorbol 12-myristate 13-acetate

PPD – purified protein derivative

PRR – pathogen recognition receptor

qRT-PCR – quantitative real time polymerase chain reaction

RAG – recombinant activating gene

RTE – recent thymic emigrant

S1P1 – sphingosine 1-phosphate receptor

SCZ – sub-capsular zone

SLC – secondary lymphoid organ *chemokine*

SP – single positive

TCR – T cell receptor

Th1 – T helper cell subset 1

TLR – toll-like receptor

TNF-α – tumor necrosis factor-alpha

WHO – World Health Organization

wpi – weeks post-infection

WT – wild type

1. *Mycobacterium* genus

The genus *Mycobacterium* houses more than 120 species [1], the great majority, non-pathogenic bacteria. However, the most well known bacteria within this genus are high virulent species such as *Mycobacterium tuberculosis* and *Mycobacterium leprea*, for example, etiological agents of tuberculosis and leprosy, respectively [2].

Exposure to mycobacteria is common worldwide, with attention normally directed to the vast number of *M. tuberculosis* infected individuals, estimated to be 1/3rd of the world's population, according to the World Health Organization (WHO) [3]. Due to the increase number in infections with the Human Immunodeficiency Virus (HIV), co-infections with *Mycobacterium avium*, an opportunist bacteria, have also gained serious interest because of the high morbidity and mortality rates in these co-infected patients [4].

1.1 Characteristics of mycobacteria

Mycobacteria are aerobic and non-motile *bacilli*, with facultative intracellular growth [5].

The mycobacterial cell wall is a very well organized structure. Right next to the cytoplasmatic membrane, mycolic acids, peptidoglycan and arabinogalactan organize themselves in a core called the mycolyl arabinogalactan-peptidoglycan complex. The outer membrane is composed by variable sized lipids and cell-wall proteins [6, 7]. The extremely low permeability of the cell wall increases the resistance capacity of these bacteria to antibiotics and chemical sanitizers [8, 9]. While the core of the cell wall is essential for bacterial viability, it's constituents are considered extremely important signaling molecules in the disease process [7, 10, 11].

The cell wall confers these bacteria with an acid-fast classification because of the ability to retain carbolfuchsin staining after discoloration with alcohol-acid solutions, and therefore considered positive for the Ziehl-Neelson stain [6]. Although mycobacteria do not seem to fit the Gram-positive category, because of the absence of an outer cell membrane, they are still classified within this group due to the lack in the ability of retaining crystal violet [12].

M. avium is a very common environmental bacteria, scattered throughout nature in soils and surface waters, but may also be isolated from tap water [2, 6]. These *bacilli* are members of the *M. avium* complex (MAC). Although bacteria from this group are normally considered as non-pathogenic, they can cause disease in certain animals and immunosupressed humans [6, 13].

1.2 The animal model of *M. avium* infection

M. avium is a slow growing bacteria, that is able to colonize and establish a bacterial growth plateau in peripheral organs such as the spleen and liver [14]. When compared with the animal model of *M. tuberculosis* infection [15-17] both kinetics of bacterial growth in infected organs are quite similar [18, 19] (Fig.1). In both infections the bacterial load increases rapidly in the first weeks of infection, however around week 3-4 bacterial growth is halted due to the initiation of an acquired immune response conducted by antigen-specific T cells with the ability to secrete interferon (IFN)-γ [20, 21].
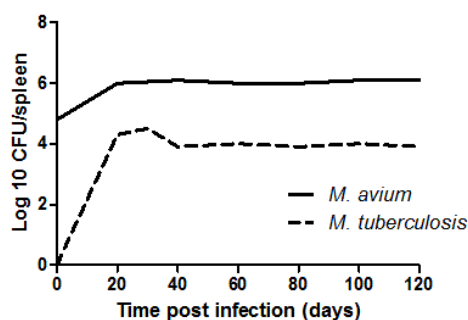
Figure 1: Course of *M. avium* and *M. tuberculosis* infection in the spleen of infected C57BL/6 mice. Colony forming units (CFU) data was adapted from Nobrega *et al.* 2007 [22] (i.v. infection with 10⁶ CFU *M. avium* strain 2447) and Cardona *et al.* 1999 [16] (aerosol infection with 100 CFU *M. tuberculosis* Erdman strain).

M. avium species can be divided into a range of different strains that can be classified according to their colony morphology and pathogenesis to the host, among other features. Particularly, intravenous infection of C57BL/6 mice with *M. avium* strain 2447 (strain used in the present work), which is an intermediate virulence strain, results in a disseminated chronic infection, during which infected animals may present no external sign of sickness for several months [23]; this strain's characteristics makes it an interesting model to study aspects of the immune response against intracellular mycobacterial infections [24, 25].

Upon intravenous (i.v.) injection, bacteria start to settle firstly in peripheral organs, such as the liver and spleen, just a few hours after infection. After 3-4 weeks of infection, bacterial growth starts to stabilize in these organs, forming a plateau that is maintained over time. The presence of this plateau is an indicator of an ongoing acquired immune response within the organ. In accordance, splenocytes from 30 day-infected mice produce higher concentrations of INF-γ, when compared with both earlier and more advanced stages of infection [26].

2. Immune response to mycobacterial infections

When entering the organism, bacteria are phagocytosed by macrophages (Mφ) and dendritic cells (DC). Mainly small proteolytic fractions are then presented to T lymphocytes, making the bridge between the innate and acquired immune response. The interplay of the two types of cells

allows activation of antigen-specific cells and its bactericidal mechanisms through the production of key molecules, such as cytokines and chemokines.

2.1 Launching the immune response against mycobacteria

Anatomical barriers, like the skin and the mucosal, act as a first barrier to the invading pathogen. After this, activation of the innate immune's inflammatory pathways and phagocytic cells begins. In infections by intercellular pathogens, such as mycobacteria, antigen presenting cells (APC), like DC and Mφ, phagocytose bacteria and present mycobacterial antigens, in the context of the major histocompatibility complex (MHC), to T lymphocytes [27].

Identification is a key factor for a fast and effective response, and for that cells have a vast variety of pattern recognition receptors (PRR), such as toll-like receptors (TLR), that recognized specific pathogen-associated molecular patterns (PAMP) like lipoarabinomannan (LAM), lipopolysaccharide (LPS), manose, CpG DNA motifs and others [28-31]. The binding with these receptors in APC leads to activation of signaling cascades that drive cell activation by production of cytokines like tumor necrosis factor (TNF)-α and interleukine (IL)-12, essential for autocrine activity and T cell activation, respectively [27].

Besides molecular identification by TLRs, opsonization mainly by immunoglobulin (Ig) G and complement molecule C3b aids in the process of phagocytosis [32]. Upon ingestion, bacteria are contained within a phagosome. During maturation, endosomes and lysosomes fuse with this compartment to increase its acidity, giving rise to the phagolysosome, and activation of enzymes that break down the up taken material [33]. Bacterial peptides are then mounted on the MHC which presents the antigen at the phagocyt's cell surface. This presentation is the point of connection between the innate and acquired immunity since the peptide-bounded MHC molecule binds to T cell receptor (TCR) on the T cell surface, along with the respective co-stimulators CD80/86 (B7.1/B7.2) and CD28/CTLA-4 [27]. Together with the binding of these receptors, DC are also responsible for the production of IL-12 which drives the differentiation of naive CD4+ T cells into a Th1 phenotype [34] for the control of intracellular pathogens, such as mycobacteria [35].

Despite all the defense machinery present in the host, virulent mycobacteria have workarounds to avoid these mechanisms [36], mainly through their ability to inhibit the formation of the phagolysosome, by preventing phagosome-lysosome fusion [36-39] and acidification [40].

2.2 The most valuable players in mycobacterial immune response

The acquired immune response steps in at a later stage of the infections (2-3 weeks upon infection) and involves a series of antigen-specific cells, such as CD4+ and CD8+ T cells, that are capable of inducing cellular activation and stopping bacterial growth through the production of pro-inflammatory cytokines, like IFN-γ [41].

For a successful initiation of the T cell response, antigen presentation needs to occur in the draining lymph node of the infection site. For this, chemokines such as EBI1-ligand chemokine

(ELC) and secondary lymphoid organ chemokine (SLC), expressed in lymph nodes, recruit both naive T cells and antigen loaded APC through the CC-chemokine receptor 7 (CCR7) [42-44].

After antigen presentation and IL-12 production, naive CD4+ T cells acquire a Th1 phenotype becoming INF-γ producers. Since T cells are essential for the control of intracellular pathogens [34] such as mycobacteria [35], IL-12, due to its Th1 phenotype inducing ability, also becomes essential for the control of both *M. tuberculosis* and *M. avium* infections [45-48]. INF-γ leads to the activation of macrophage's bactericidal mechanisms being one of the most relevant in the production of nitric oxide (NO) upon activation of the inducible nitric oxide synthase (iNOS). This enzyme is a marker for APC activation, and although iNOS production by Mφ is a key element for controlling *M. tuberculosis* infection [20], this is not the case for infection by *M. avium* [23, 49] since its growth is not impaired in mature phagolysosomes, even after fusion with the acidified vacuoles [50].

Together with autocrine production of TNF-α by Mφ, INF-γ are both key molecules for the control of mycobacterial infections [51]. Genetically modified mice that lack the ability to produce either one of these cytokines have been shown to be more vulnerable to *M. tuberculosis* [52-55] and *M. avium* infections [21, 56]. In addition to INF-γ and TNF-α, several receptors and molecules are essential for controlling the bacterial burden. The lack of a single key player may lead to loss of control in the immune response and increase in bacterial proliferation (Fig.2).
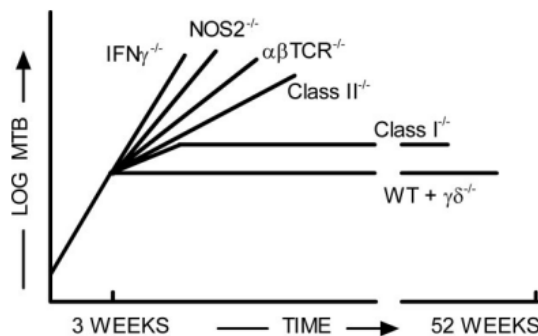
Figure 2: Bacterial progression of M. tuberculosis infection in the lung of wildtype (WT) and genetically altered mice strains. Aerosol infected mice (100 *M. tuberculosis* H37Rv CFU/mouse) have equal bacterial growth until initiation of an acquired immune response (3 weeks). After this, that lack of key molecules such as INF-γ and NOS2 proves to be fatal to mice lacking these genes. Increased susceptibility is also present in αβTCR and MHC classe II knockout (KO) mice, when compared with WT and γδ KO mice. Figure from North *et al.* 2004 [57].

While cytokines are responsible for activation or inhibition of specific cells or cellular functions, chemokines are in charge of guiding these cells to the regions where they are necessary [58], and can be produced not only by immune cells, but also by, for example, lung resident cells, such as epithelial, stromal and endothelial cells, to attract both Th1 and Th2 cells [59].

Every cell has its collection of receptors that interact with different ligands and that also distinguish them from other cells. For example, Th1 cells express specifically the CXC-chemokine receptor 3 (CXCR3) which reacts to the inflammatory chemokines interferon-inducible protein-10 (IP-10), monokine induced by gamma-interferon (MIG) and interferon-inducible T cell alpha chemoattractant (I-TAC) [60-62].

The presence of chemokine receptors can also be a marker for a cell's differentiation state: naive CD4+ T cells express the receptor CCR7, which improves the ability to interact with the constitutive chemokines ELC and SLC, expressed within lymph nodes [42-44]. However after differentiating, CD4+ T cells up-regulate a series of receptors, including CCR5 and CXCR3, that enhance their ability to react with inflammatory chemokines, such as macrophage inflammatory protein-1β (MIP-1β), MIG, IP-10 and I-TAC [60-62].

Chemokines from the MIP family have been shown to be important in mycobacterial infections in a vast variety of models. Levels of macrophage inflammatory protein-1α (MIP-1α) and MIP-1β are increased in bovine alveolar Mφ infected with *M. tuberculosis*. IL-8 is also elevated in the case of *Mycobacterium bovis* infected bovine alveolar Mφ [63] and monocyte derived Mφ from HIV patients co-infected with MAC disease [64].

Production of these chemokines has been shown to be activated by intracellular bacterial growth, but not virulence or growth rate [65]. Increased expression of MIP related chemokines and IL-8 enhances the capability of the host to recruit monocytes [64], several types of granulocytes [63], such as neutrophils [66]. Beige mice have been shown to be susceptible at early stages of *M. avium* infection when compared with WT mice. This is coincident with a diminished expression of MIP-1β and MIP-2 in the lung at early stages of infection [67].

Th1 attracting chemokines have an extreme importance in mycobacterial infections [68]. T cells retrieved from the draining mediastinal lymph nodes of mice injected with *M. bovis* purified protein derivative (PPD) have increased levels of CXCR3 and decreased levels of CCR7 [69]. While CXCR3+ cells are associated with a T cell activated phenotype, CCR7+ T cells are related to memory phenotype, however CCR7 is downregulated after stimulation with PPD, gaining a CXCR3+ phenotype [69]. Moreover, *Mycobacterium ulcerans* [70] and *M. tuberculosis* [71] infected mice have increased levels of the three major Th1 attracting chemokines, MIG, IP-10 and I-TAC when compared with non-infected animals. Elevated levels of these chemokines are also visible in DC [72] and serum [73] from *M. tuberculosis* infected patients.

As T cells originate from the thymus, the structure and function of this primary lymphoid organ shall be explored below.

3. Structure and function of the thymus

T lymphocytes are generated throughout life, arising from bone marrow (BM)-derived progenitors that complete an essential developmental process within a specialized structure and microenvironment: the thymus. Thymic T cell education leads to the generation of a self-restricted (that are capable of recognizing self MHC molecules) and largely self-tolerant (non-reactive with self-antigens) peripheral T-cell pool, with a great variety of TCRs and therefore a large spectrum of action.

The thymus is responsible for the differentiation of T lymphocytes, which are critical cells of the acquired immune system. This organ is formed by an outer zone, the cortex, an inner zone, the medulla, and the cortico-medullary junction (CMJ), each of them capable of creating a specialized microenvironment able to differentiate precursor cells that arrive from the bone marrow. Differentiation of thymocytes in this organ can be followed by using several surface markers [74-77] (Fig.3).
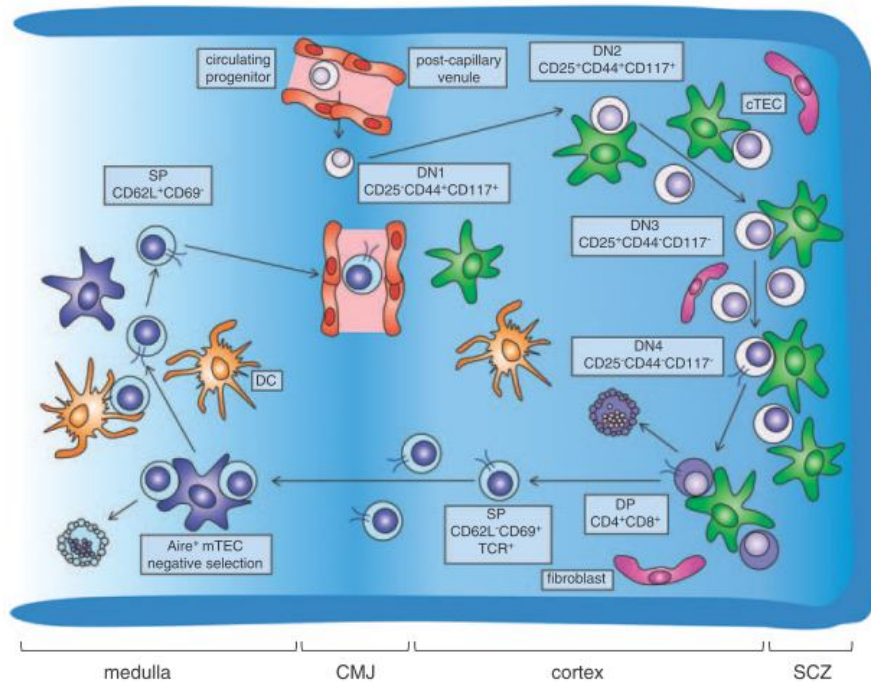
Figure 3: Thymocyte migration and differentiation within the thymus. Circulating progenitors enter the thymus through the cortico-medullary junction (CMJ) to start their differentiation process. These double negative (DN) cells, that do not express either CD4 nor CD8, migrate to the cortex and sub-capsular zone (SCZ) were they undergo 4 different DN (DN1 to DN4) maturation stages by interacting with cortical thymic epithelial cells (cTEC) and combining up/down regulations of both CD25 and CD44. Pre-T cell receptor (TCR) is expressed at the end of DN maturation, followed by co-expression of CD4 and CD8, along with CD3, becoming double positive (DP) cells. These cells interact with antigens presented by MHC on the surface of cTEC, in the process of positive selection, to test their binding capacity. Those that are able to recognize self-MHC then migrate back to the medulla and choose one of two phenotypes: CD4 or CD8 single positive (SP) thymocytes, that express also CD69. In the medulla, SP thymocytes undergo a negative selection process, interacting with medullary thymic epithelial cells (mTEC) and dendritic cells (DC), and those whom TCRs bind with high affinity to self-antigens eventually die. Cells that survive negative selection, down regulate CD69 and express CD62L, migrating back to the CMJ, expressing the chemokine receptors CCR7, CCR9 and S1P1 which will aid them in exiting the thymus and re-entering circulation. Figure from Bunting *et al.* 2011 [78].

A critical molecule in T cell differentiation is the TCR. This heterodimer receptor is composed by an α and β chain (γ and ∂, in the case of γ∂ T cells). The diversity of the peripheral T cell pool is due to the random gene arrangement of VJ (α chain), V(D)J (β chain) [79] and to the three hyper-variable complementarity determining regions (CDR) [79, 80], present on each chain. Throughout differentiation, the TCR is challenged in several occasions, being each binding essential in T cell fate decisions.

Circulating progenitors enter the thymus through the cortico-medullary junction (CMJ) after leaving the BM and express neither CD4 nor CD8, and therefore are called double negative (DN; CD4·CD8·) cells; since they do not express the T cell marker CD3, they can also be referred to as triple negative (CD3·CD4·CD8·) cells. These cells can be further divided into four cell subsets

according to their CD25 and CD44 expression: DN1, DN2, DN3 and DN4. DN1 have high expression of CD44 but no expression of CD25. At this stage, they migrate through the cortex into the sub-capsular zone (SCZ), interacting with cortical thymic epithelial cells (cTEC), fibroblasts and Mφ as they pass, further differentiating into DN2 cells (CD25+CD44+) [81-83]. Upon entering the SCZ, thymocytes lose expression of CD44, entering the DN3 stage, where recombination of TCR genes begin. With the assembling of a TCRβ chain and a pre-TCRα chain, together with a low expression of CD3, thymocytes commit themselves to the αβ lineage, further differentiating into DN4 cells (CD25-CD44-CD117-) [84]. At this stage the pre-TCR expressed alongside with co-expression of CD4 and CD8, allows cells to re-route their destination to the medulla, becoming double positive (DP, CD4+CD8+) cells. While passing through the cortex, TCRα chain is rearranged and the TCR's ability to bind with MHC molecules is tested [85, 86]. Thymocytes expressing TCRs with intermediate affinity/avidity for peptide-MHC complexes [expressed by cortical thymic epithelial cells (cTEC), DC, fibroblasts] receive survival signals resulting in positive selection for thymocytes with self-MHC-restricted TCRs, while those with higher and lower affinity/avidity die, by negative selection or by neglect, respectively [87].

Cells able to recognize self-peptide-MHC complexes then migrate to the medulla and differentiate into one of two phenotypes: CD4 or CD8 single positive (SP, CD4+CD8- or CD4-CD8+, respectively) thymocytes, that express also CD69. These cells then enter the process of negative selection, mediated by interactions with self-antigen-presenting medullary thymic epithelial cells (mTEC) and DC. SP thymocytes expressing a TCR with high affinity/avidity for self-antigen are deleted during negative selection, reducing the likelihood that self-reactive T cells will enter circulation. SP cells that survive negative selection, down regulate CD69 and express CD62L, migrating back to the CMJ, where they exit the thymus and re-enter circulation now as naive CD4+ or CD8+ T cells [87]. Besides this, exiting T cells have a high expression of CD24 and low expression of CD44 [88].

To fully finish their differentiation process, T cells when exiting the thymus, express on their surface CCR7, CCR9 and sphingosine 1-phosphate receptor (S1P1) [78]. These chemokines receptors guide newly generated T cells to lymph nodes where they can fully differentiate by binding to specific-antigen APC [42-44]. After this, T cells downregulate these receptors upregulating receptors that are able to interact with chemokines usually expressed during infections or inflammatory stimuli [60-62].

Newly differentiated T cells after finishing maturation in the thymus, are ready to exit the organ, entering in circulation, therefore come to be called as recent thymic emigrants (RTE). Neonatal RTE are essential for peripheral colonization and diversity in early stages of life, and are functionally more efficient than adult RTE [89], producing more effector cytokines like IL-2, -4 and INF-γ and proliferate more after IL-7 stimulation [89].

Adult RTE are considered important to fill in the gaps left by dying cells and, therefore, maintain the peripheral naive T cell pool diversity. However, adult RTE are considered to be immature and lacking full functional capacity when compared with mature peripheral T cells [90], which may or

not be coincidence with the fact that the adult thymus undergoes a physiological involution, with progressive loss of activity [91-94]. Therefore, although commitment to T cell lineage is decided within the thymus [95], extrathymic activity is essential for continuation of T cell maturation [96-99] and T cell repertoire shaping [100-102].

4. The thymus as a target of infections

Given that the thymus is a primary lymphoid organ, it is not frequently seen as a possible target of infections. The thymus has been studied in the context of several infections but not as a possible site for an immune response. Mouse models have been shown to shelter protozoa [103-107], bacteria [108] such as the ones from *Mycobacterium* species [22, 109, 110], fungi [111] and viruses [112-119].

In humans, thymic infection has also been demonstrated, especially in HIV infections [120-123]. On the contrary, only a few case reports have described infection of the thymus with mycobacteria [124-131].

The first report of an animal model of thymic infection appeared in 1968, however it was not adequate to study the chronic outcome of this infection due to the lethal dose of injected bacteria [109, 110]. To better analyze this issue, our group was able to utilize an murine model, able to develop chronic disease, to study thymic infection [22]. Intravenous infection with *M. avium* established a plateau of bacterial growth within the thymus, but at a later time when compared to the spleen (Fig.4). This was also the case for aerosol infection with *M. tuberculosis* and intravenous infection with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG).
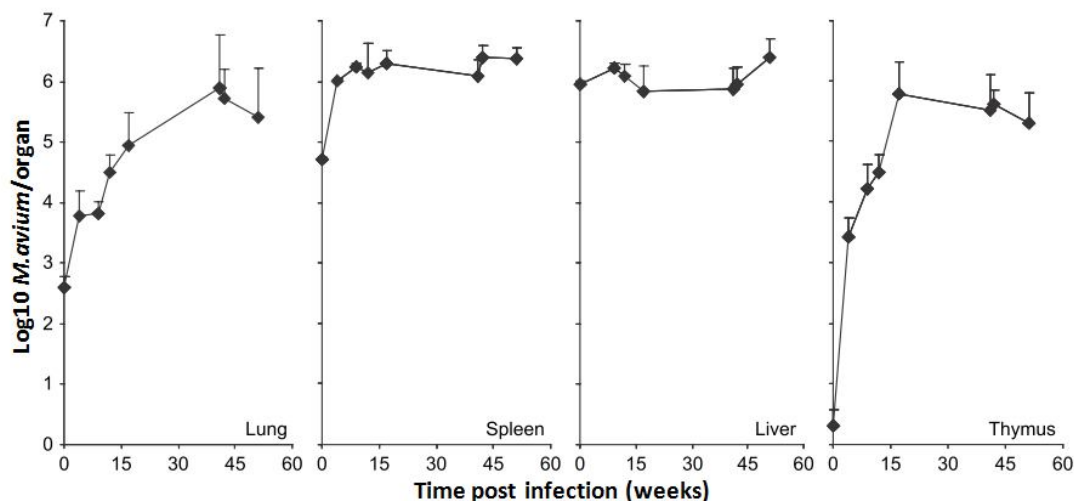
Figure 4: Bacterial progression in the organs of infected mice. When compared with the lung, liver and spleen, more time is necessary for the thymic bacterial load to reach the sames levels as peripheral organs. A bacterial plateau is well established in the *M. avium* mouse model of infection, however, it only occurs after 16 weeks of infection, while in the spleen, bacterial growth is halted around 4 weeks post-infection. C57BL/6 mice were infected intravenously with 10^6 CFU of *M. avium*. Figure from Nobrega *et al.* 2007 [22].

4.1 The outcome of mycobacterial infections in the thymus

Upon experimental infections, the thymus has been shown to be affected by several different microorganisms, but also not associated with organ colonization. A frequent characteristic of these infections is a visible thymic atrophy, revealed by weight and cellular loss, in general owed to loss of cortical thymocytes [Table 1], mainly through DP depletion. Atrophy could be directly related with infection in the case of *Trypanosoma cruzi*, since this phenomenon has been shown to be reverted by decreasing parasitaemia [132], benznidazole treatments (used against *T. cruzi* infections) [133] or blockage of key enzymes of the parasite [134].

Host aspects, induced by infection, are also involved in thymic atrophy. In some cases, infected animals with thymic atrophy had elevated corticosteroids levels [108, 135], which could be reverted by inhibition of corticosteroid signaling through adrenalectomy or blockage of corticosteroid receptors reverted thymic atrophy [108, 135]. It is also thought that TNF may be involved in thymocyte apoptosis signaling since atrophy was not observed in mice deficient for TNF-receptors infected with *Francisella tularensis* [108, 136].

Table 1: Thymic atrophy in different animal models of infection.

	Infectious Agent	Model	Cortical atrophy[1]	DP depletion	Reference
Protozoa	*Trypanosoma cruzi*	Mouse	+[2]	+	[103-105]
	Plasmodium berghei	Mouse	+	+	[106, 107]
Bacteria	*Francisella tularensis*	Mouse	+/-[3]	+/-[3]	[108]
Fungi	*Paracoccidioides brasiliensis*	Mouse	+	n.a.	[111]
Virus	Murine Leukemia virus	Mouse	+	+	[112, 113]
	Measles virus	SCID-hu mouse	+/-[3]	+/-[3]	[114]
	Simian Immunodeficiency Virus	Macaques	+/-[3]	+/-[3]	[115]
	Human Immunodeficiency Virus	SCID-hu mouse	+	+	[116-119]

n.a.: not assessed; DP: double positive CD4·CD8· thymocytes; SCID: Severe combined immunodeficiency; [1] Assessed histologically; [2] Reverted by pathogen clearance; [3] Dependent of infectious agent strain.

Since T cell differentiation is dependent of the thymus, the impact of infection on the generation of mycobacteria specific T cells has been explored by our lab. Our group has shown that T cells originated from infected thymi are impaired in the ability to produce INF-γ in response to mycobacterial antigens but not when stimulated with a mitogen [such as concanavalin A (ConA)] or with unrelated antigens to mycobacteria such as ovalbumin (OVA) upon immunization [137]. This meaning that, during infection with *M. avium*, infected cells within the thymus induce tolerance specifically to mycobacterial antigens.

Tolerance occurs when the immune response is well established in the periphery, and therefore may have no significant impact in peripheral organs. Supporting this idea is unpublished data from our lab that show that mice whose thymus was infected have no alteration in peripheral

bacterial proliferation after thymectomy. However, in cases of peripheral lymphopenia, such as co-infected HIV patients, this situation might be extremely relevant due to the gradual loss of specific mycobacterial T cells that cannot be recovered by competent newly generated cells by the thymus.

In addition to alterations on T cell differentiation, we hypothesize that an immune response is taking place within the thymus, since the bacterial load is halted in this organ about 16wpi [22]. Interestingly, this stoppage in the bacterial growth occurs at later time points when compared with the spleen and liver. Since this standstill of the bacterial burden in the spleen occurs at 4wpi, and being this arrest associated with an increase in INF-γ production, we hypothesize that the same may happen in the thymus.

Knowing how the cytokine profile is altered within the thymus is of relevance, not just to characterize the mycobacterial immune response within this organ, but also because cytokines, when produced in the thymus, are known to play a different role, during T cell differentiation, and in the periphery during an immune response to infection [138].

Interestingly, pro-inflammatory cytokines like INF-γ, have been described to be produced in the thymus of healthy animals and have a distinct effect, essentially responsible for the development of T lymphocytes [138]. In peripheral organs, such as the spleen or lymph nodes, this cytokine has a crucial effect on bacterial proliferation and APC activation, as previously described. IL-7, is also known to be a active participant in T cell development [139]. Mice lacking the ability to produce this cytokine or its receptor have a significant reduction in $\alpha\beta$ lymphocytes and a complete lack of $\gamma\partial$ lymphocytes [140-142]. Preliminary data from our laboratory illustrates an altered cytokine profile and bacterial growth plateau in the thymus, when compared to the peripheral organs like the spleen or the liver.

As INF-γ producing T cells are considered to be the major conductors of the acquired immune response against mycobacteria, we speculate of their involvement in the thymic infection. Our previous results show that T cells that develop in an infected thymus come out tolerant when responding specifically to mycobacterial antigens. Although the mechanism for the induction of this tolerant has not yet been discovered, we could not discard the possibility that these are simply staying in the thymus, since they have already found a site of infection. However, this assumption is contradictory, since newly differentiated T cells are considered to be functionally defective in comparison with circulating mature T cells in their ability to produce INF-γ [89, 90].

Consequently, it becomes difficult to consider these newly differentiated cells to be active producers of INF-γ and therefore it should be taken into consideration that circulating cells may be responsible for controlling the infection, as major producers of pro-inflammatory cytokines. In infected organs, such as the liver and spleen, intervening T cells arrive from the peripheral pool of mature cells, and so, this may also be valid for the thymus. It is known that cells re-circulate back to the thymus, however, why they do so is still unknown [88, 143-145]. If these re-circulating cells are participating in the immune response, it is possible that this circulation back to the thymus is due to a simple surveillance routine.

In the current proposal we intend to investigate the characteristics of the immune response against mycobacteria within the thymus:

- Characterize the cytokine and chemokine profiles of infected thymi compared to those established in peripheral infected organs like the spleen and lung;

- Evaluate to what extent RTE and/or re-circulating mature T cells are responsible for the immune response against mycobateria within infected thymi.

Mice – C57BL/6 (wild-type [WT]) mice were purchased from Charles River Laboratories (Barcelona, Spain). RAG2p-GFP (RAG-GFP) and TCRα−/− (TCR−/−) mice were obtained from crossings preformed at the ICVS animal facilities being the initiating couple kindly provided by Dr. A. Bandeira Ferreira (Institut Pasteur, Paris, France) and purchased from The Jackson Laboratories (Bar Harbor, USA), respectively. 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*.

Experimental infection – Eight-week-old female mice were infected *i.v.* with 10^6 CFU *M. avium* strain 2447 (provided by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium). At specific time-points mice were sacrificed by decapitation or CO_2 inhalation, for bacterial load determination, organs were removed in aseptic conditions, homogenized in distilled sterile water with 0.05% Tween 80 and serial dilutions were prepared and plated onto Middlebrook 7H10 agar medium. Plates were incubated for 1 week at 37°C and the number of CFU counted.

Gene expression analysis – Thymi, spleens and lungs were harvested from both non-infected and infected animals and stored at -80°C until extraction. Total RNA was isolated using TRIzol® Reagent (Invitrogen) and reverse transcribed into cDNA using the superscript first-strand synthesis system for reverse-transcription polymerase chain reaction (PCR) (Invitrogen) according to manufacturer's instructions. Expression levels of all genes were assessed by quantitative real-time PCR (qRT-PCR) using SsoFast™ EvaGreen Supermix® (BIO-RAD) in a BIO-RAD CFX96™ Real-Time System with a C1000™ Thermal Cycler. The hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as reference gene. Specific oligonucleotides were used for *HPRT* (sense: 5'-GCT GGT GAA AAG GAC CTC T-3'; antisense: 5'-CAC AGG ACT AGA ACA CCT GC-3'), *IFN-γ* (sense: 5'-CAA CAG CAA GGC GAA AAA GG-3'; antisense: 5'-GGA CCA CTC GGA TGA GCT CA-3'), *iNOS* (sense: 5'-CTC GGA GGT TCA CCT CAC TGT-3'; antisense: 5'-GCT GGA AGC CAC TGA CAC TT-3'), *IL-10* (sense: 5'-AGG ACT TTA AGG GTT ACT TGG GTT-3'; antisense: 5'-GCT CCA CTG CCT TGC TCT TAT T-3'), *MIG* (sense: 5'-CTT TTC CTC TTG GGC ATC AT-3'; antisense: 5'-GCA TCG TGC ATT CCT TAT CA-3'), *IP-10* (sense: 5'-GCT GCC GTC ATT TTC TGC-3'; antisense: 5'-TCT CAC TGG CCC GTC ATC-3'), *MIP-1β* (sense: 5'-GCC CTC TCT CTC CTC TTG CT-3'; antisense: 5'-GAG GGT CAG AGC CCA TTG-3') and *IL-7* (sense: 5'- CGC AGA CCA TGT TCC ATG T-3'; antisense: 5'- TCT TTA ATG TGG CAC TCA GAT GAT-3'). The cycling parameters were one cycle of 95°C for 1 min, one cycle of 95°C for 15 s, 40 cycles of optimal temperature for 20 s plus 72°C for 20 s and one cycle at 65°C for 5 s. Optimal temperatures were 57°C for *MIG* and *MIP-1β*, 58°C for *HPRT*, *IFN-γ* and *IP-10* and 59°C for *iNOS* and *IL-10*. Relative expression was calculated using HPRT as a reference gene and the relative expression ratio equation with standard curve method.

Immunofluorescence – Sections (7 mm) of paraffin embedded thymi were hydrated and antigen retrieval was performed by heating the slides in EDTA 1mM for 30min at 96ºC during and then cooling them to room temperature for 20min in the same solution. Unspecific binding was blocked using 4% BSA in PBS 0.05% Tween. Tissues were incubated overnight at 4°C with primary antibody (Ab) [rabbit polyclonal IgG anti-mouse iNOS (Santa Cruz Biotechnology)]. Secondary Ab used was goat anti-rabbit IgG Alexa Fluor 488 (Molecular Probes) and nuclei were stained using DAPI (Vector Laboratories, Inc.). Mycobacteria were detected by auramine-rhodamine (A&R) staining (AlphaTec Systems) previous to the incubation with the primary antibody. Slides were visualized using an upright confocal microscope (Olympus FV1000), and images were analyzed using FV10-ASW 2.0 Viewer software (Olympus). All stainings were optimized and no specific signal was observed in negative controls (iNOS knockout mice) or incubation with secondary Ab. No signal was observed on non-infected tissues stained for A&R.

Flow cytometry – Cell suspensions of thymus and spleen were prepared by mechanical disruption with two notched slide glasses. Cells were resuspended in DMEM (Gijbco) supplemented with penicillin-streptomycin, L-glutamine, sodium pyruvate, fetal bovine serum (FBS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, all from Gijbco). $5x10^6$ thymocytes or $2x10^6$ splenocytes were labeled with specific Abs for CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD24 (M1/69), CD44 (IM7, all from BioLegend) and tetramer for Ag85 (FQDAYNAAGGHNAVF, Tetramer Core Facility, Emory University).

For intracellular staining, $2x10^6$ cells were incubated with the combination of phorbol 12-myristate 13-acetate 200ng/ml and ionomycin 2000ng/ml (PMA+ION) or Ag85 40µg/ml for 7hours, being the last 5 in the presence of brefeldin at 40µg/ml. PMA+ION was used as a positive control while culture medium was used as a negative. Cell were then washed and surface staining was preformed. Suspension was then fixed with 2% formaldehyde for 20min in the dark at room temperature. After being washed, cells were permeabilized for 15min at room temperature with 0.5% saponine. Unspecific binding was blocked using anti-CD16/32 antibody (93, BioLegend) and cells were then stained for IFN-γ (XMG1.2, BioLegend) during 30min at room temperature.

Cell acquisition was performed on a LSRII flow cytometer (BD Biosciences) using the FACS Diva Software (BD Biosciences). FlowJo Software (Tree Star) was used for data analysis, doublets were excluded based on a FSA-A vs. FSA-H plot.

Cell purification and Cell sorting – CD4 cells from thymus and spleen were purified by magnetic labeling using a CD8α Microbeads (cat. no. 130-049-401) or CD4+ T cell isolation kit (cat. no. 130-095-248), respectively. Staining for magnetic separation was performed according to the manufacturer's instructions, except for the amount of each kit component used for thymocyte purification (double the one recommended). Separation of magnetic labeled cells was performed on an autoMACS Pro Separator (Miltenyi Biotec). Afterwards, cells were stained and the populations of interest were sorted on a FACSAria cell sorter.

Statistical analysis – Differences among the means of experimental groups were analyzed using the two-tailed Student *t* test. Differences with a *P* value ≤0.05 were considered significant. Significance was referred to as * when P≤0.05, as ** when P≤0.005 and as *** when P≤0.001.

For gene expression levels, the ratio of gene's mean expression in infected mice over non-infected mice is represented. Represented significance of these graphs came from previous comparison of the two groups using the two-tailed Student *t* test.

1. Activation of antigen presenting cells in the thymus increases throughout infection

Infected macrophages, if appropriately activated, up-regulate the expression of iNOS. Thus, to investigate if infected cells within the thymus are activated we have analyzed thymi from mice infected with 12, 16 and 24 weeks for the expression of iNOS, by immunofluorescence. iNOS is normally expressed within the thymus [146, 147] and is associated with cell-mediated apoptosis that takes place [148-150]. For this reason, it is important to compare the presence of iNOS staining in non-infected versus infected thymi. Although more common in infected tissues, all non-infected thymi also prove to be active, showing iNOS expression (Fig.1A), but lower when compared with infected tissue. No increase in iNOS staining in non-infected thymi is obvious with the age of the animal (data not shown). Few bacteria are visible in the thymus at 12wpi and so iNOS expression is very low, being the great majority of the infected cells are iNOS- (Fig.1B, white arrows). As the bacterial load increases within the thymus, iNOS expression is also progressively more evident (Fig.1C, D). At 24wpi, the great majority of the infected cells are iNOS+, which gather in characteristic cellular infiltrates (Fig.1E-G). Interestingly, iNOS expression was not detected in some of the infected cells (Fig.1F, white arrows).
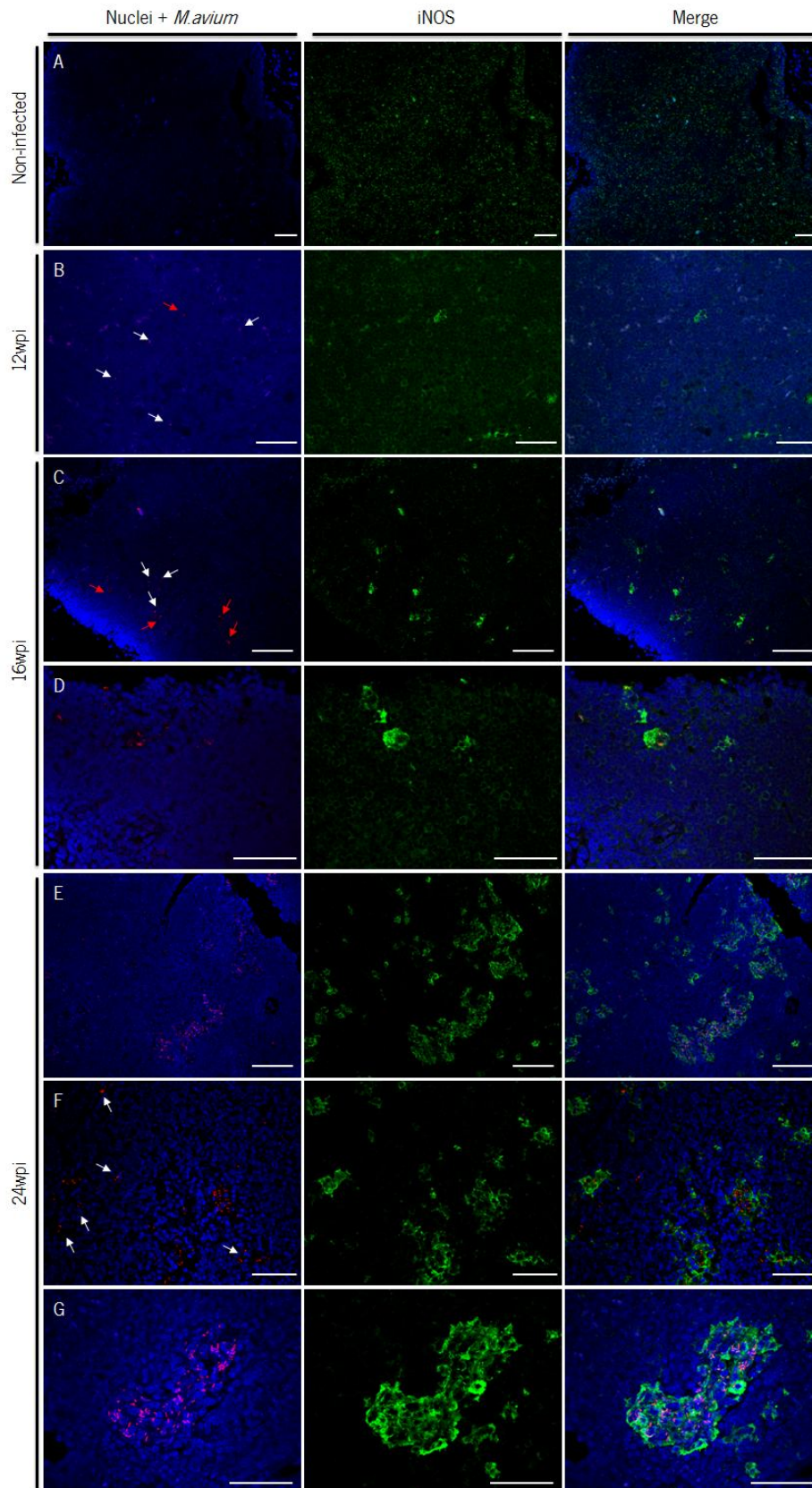
Figure 1: APC activation in the thymus assessed by iNOS staining increases over time with the bacterial load. Representative thymic sections form non-infected (A) and *M. avium*-infected WT mice after 12 (B), 16 (C, D) and 24 weeks post-infection (wpi) (E-G). All slides were stained with a specific Ab for iNOS (green) and fluorescent dyes: DAPI for nuclei (blue) and auramine-rhodamine for *M. avium* (red). Mycobacteria were detected in all infected slides starting from 12wpi. White arrows represent infected cells with no expression of iNOS while red arrows represent *M. avium* infected iNOS· cells. Pictures were obtained from 3 to 4 thymi per group, from 3 independent experiments. Bar = 50000µm.

2. Late immune activation profile in the thymus comparing with the spleen and lung

Since the stabilization of the bacterial burden in the spleen is coincident with the increased production of IFN-γ by splenocytes [26], we hypothesized that this might also occur in the thymus. In the thymus, bacterial growth is halted around 16wpi and, taking into account what happens in the spleen, the cytokine profile was analyzed in this organ. Since the bacterial load in the lung has a very slow progression, similar to the thymus, cytokine assessment was also analyzed in this organ.

Sustained increase of IFN-γ and iNOS mRNA levels were detected right from 4wpi in the spleen of infected animals in comparison to non-infected mice, while IL-10 was only transiently detected at 12wpi (Fig.2). This was also the case for IFN-γ in the lung of infected animals, but curiously, although increased expression was visible since 4wpi, iNOS expression only occurs at 24wpi, whereas a sustained IL-10 increased expression is observed from 12wpi (Fig.2). The same increased pattern of IFN-γ was visible in thymus only from 16wpi further, followed by an increased expression of iNOS at 12wpi, while no difference was observed in the expression of IL-10 (Fig.2).

As IL-7 is a key cytokine within the thymus, essential for T cell differentiation, its expression was also measured in the context of the infection. No alteration was observed in infected animals in the expression of this cytokine in the thymus throughout infection when compared with non-infected mice (Fig.3).

Levels of TNF-α, IL-1β and IL-12p35 were also analyzed in all organs, however, results of independent experiments were not consistent, and therefore will be object of future work.
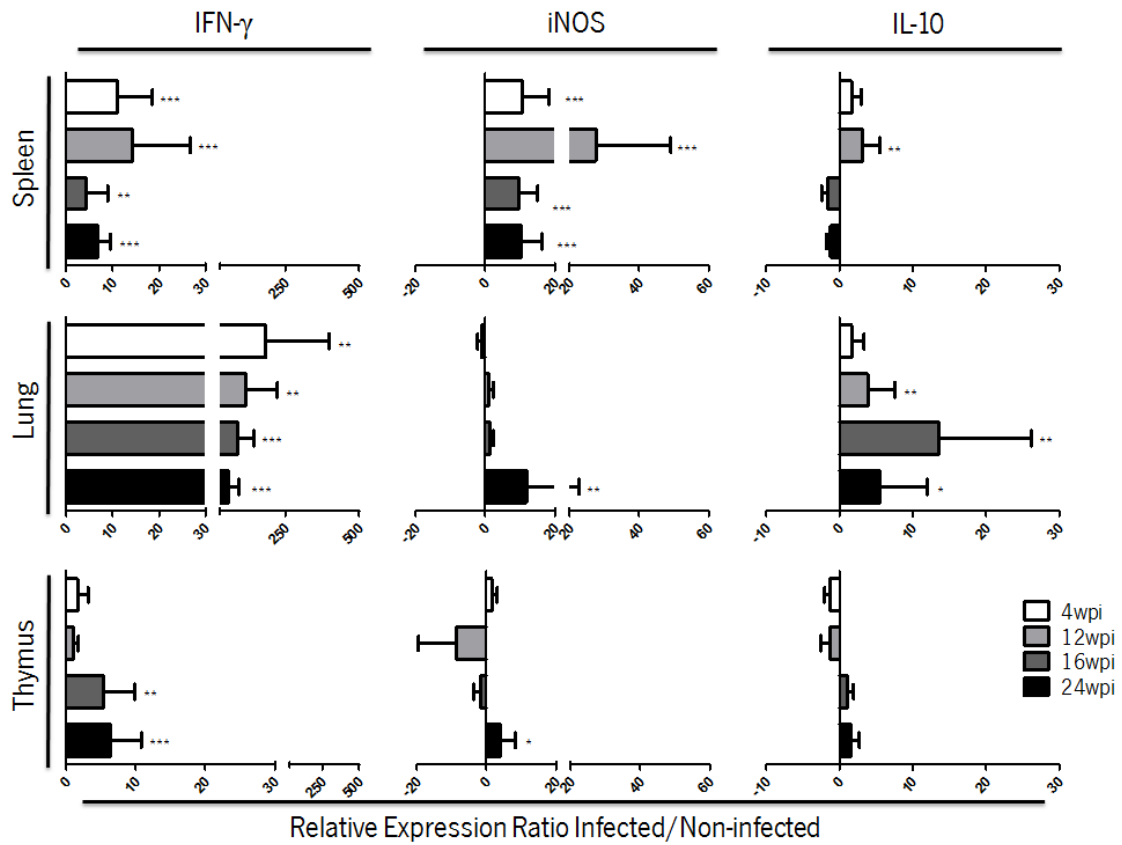
Figure 2: Increased levels of IFN-γ are coincident with the stabilization of the bacterial burden. Each column represents the mean and standard deviation of the ratio infected/non-infected with 7 to 9 mice per group from one out of two independent experiments. Significance was referred to as * when P≤0.05, as ** when P≤0.005 and as *** when P≤0.001.
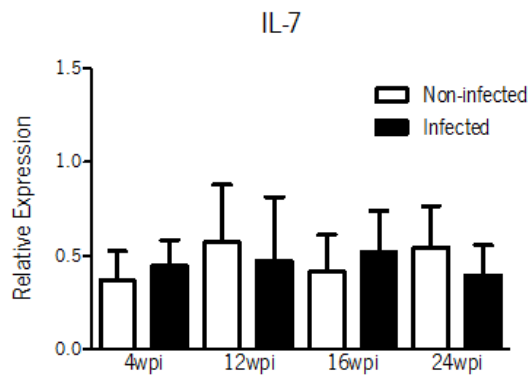
Figure 3: Infection in the thymus does not alter the expression of IL-7 over time. Each column represents the mean and standard deviation of 7 to 9 mice per group, from one out of two independent experiments.

3. Chemokines responsible for attracting Th1 cells are expressed in the thymus during infection

T cell that upon activation within the lymph node develops a Th1 phenotype, expresses on their surface the CCR5 and CXCR3 receptors. These molecules enhance the cells' ability to react with chemokines like MIP-1β, IP-10 and MIG. The expression of these chemokines in infected tissues increase the recruitment of Th1 T cells [60-62].

As expected, the expression of these Th1 cell recruiting chemokines IP-10, MIG and MIP-1β is increased in the spleen and lung as early as 4wpi. The expression of these chemokines occurs also in the thymus, from 16wpi further (Fig.4).

Figure 4: Infection increases the expression of Th1 recruiting chemokines. Each column represents the mean and standard deviation of the infected/non-infected ratio with 7 to 9 mice per group, from one of two independent experiments. Significance was referred to as * when P≤0.05, as ** when P≤0.005 and as *** when P≤0.001.

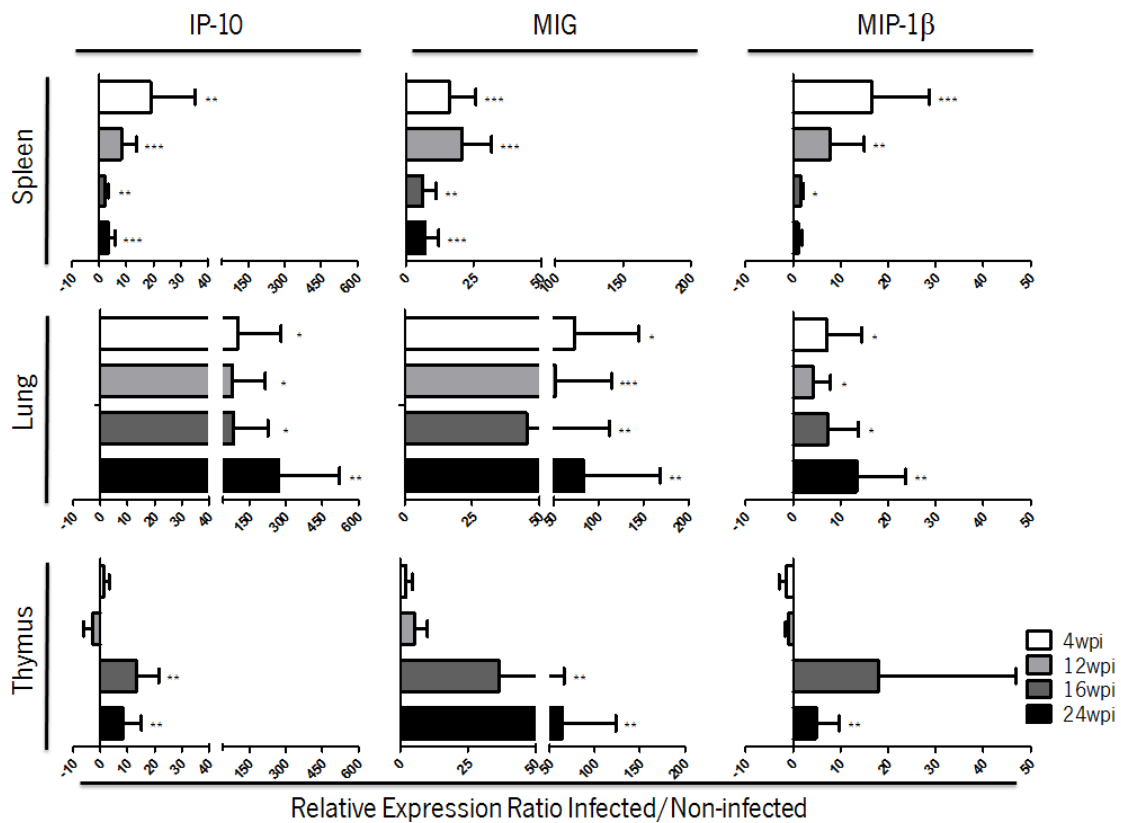## 4. Recruitment of mycobacterial specific T cells is enhanced in the infected thymus

Mice expressing GFP under the control of the RAG2 promoter represent an animal model that enables the discrimination of RTEs from mature T cells. During differentiation, RAG2 is only expressed during the rearrangement of TCR. Even though RAG2 is down regulated after thymic selection, cells are kept GFP+ for about 2 weeks [96]. Therefore, this model permits a distinction, within the thymus, of newly differentiated T cells (GFP+) from T cells that are re-circulating (GFP-) from the periphery [88]. This model has enabled the characterization of re-circulating cells in the thymus, telling them apart from cells that are undergoing differentiation.

Re-circulating T cells (GFP) by surface markers; these cells are CD44hi CD24lo [88]. To confirm that the expression of these two markers, among SP thymocytes, might be used to discriminate re-circulating T cells in wild-type mice, we have analyzed to what extent cells that are CD44hi CD24lo (Fig.5A, upper figures) were coincident with GFP- CD4 SP thymocytes (Fig.5A, lower figures). When comparing the analysis between CD44+CD24- cells and GFP- cells, we observed that the great majority of these last cells had indeed an CD4+CD24- phenotype, enabling the use of both types of analysis. Therefore, when analyzing the frequency of re-circulating cells within the CD3+CD4+ pool with both approaches of analysis, we observed no significant difference between each (Fig 5B).

This result allowed us to extend the analysis of re-circulating T cells through the thymus in WT mice using the CD44 and CD24 surface markers. In these mice, infection did not alter the number of CD44+CD24- cells being recruited from the periphery into the thymus at different time points (Fig. 5C). This was also confirmed in the RAG-GFP model at 20wpi (Fig 5B).
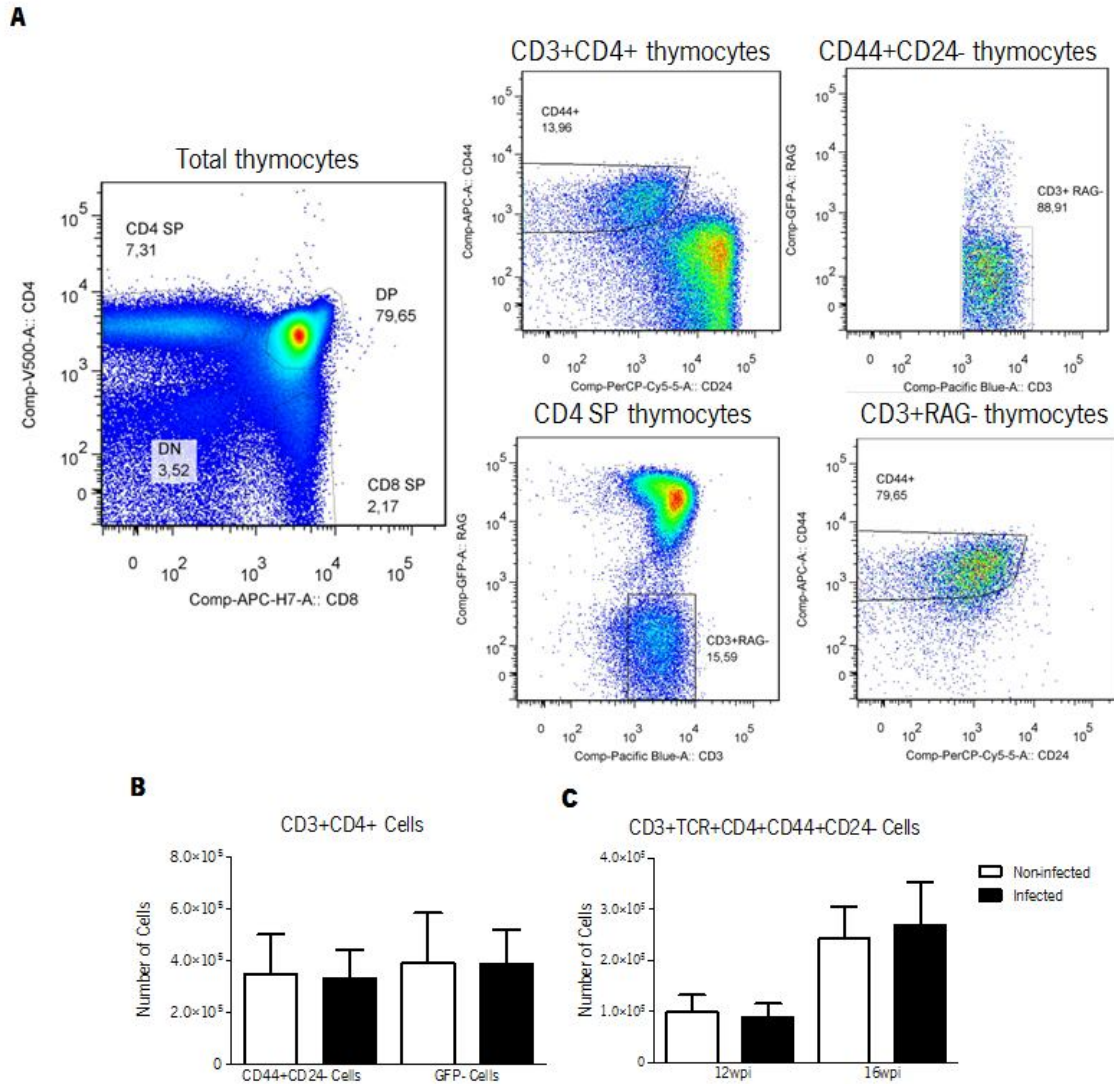
Figure 5: Infection does not affect recruitment of T cells from the periphery into the thymus. (A) Representative scheme of the cytometry analysis of re-circulating mature T cells in the thymus of RAG-GFP using the combination of CD44 and CD24 markers and RAG-GFP. (B) Comparison of re-circulating T cells in 20 weeks post-infection (wpi) in RAG-GFP mice identified by using the markers CD44 and CD24 or by the absence of GFP. (C) Analysis of the number of re-circulating cells in the thymus of WT mice at different times of infection. Each column represents the mean and standard deviation of 6 to 9 mice per group.

To analyze the specificity of re-circulating T cells in the thymus, cells were incubated with tetramers copulated with the mycobacterial epitope antigen 85 (Ag85). Analysis of re-circulation of mycobacteria-specific T cells was performed in both RAG-GFP (Fig.6A) and WT animals. Infected thymi have an increased number of tetramer-positive cells with peripheral origin after 16 weeks of infection in WT mice (Fig.6B) and also in transgenic RAG-GFP mice with 20wpi (Fig.6C). This data shows that peripheral T cells that are re-circulating back to the thymus are more enriched with mycobacterial epitope Ag85-specific cells, even though total re-circulation is not augmented.
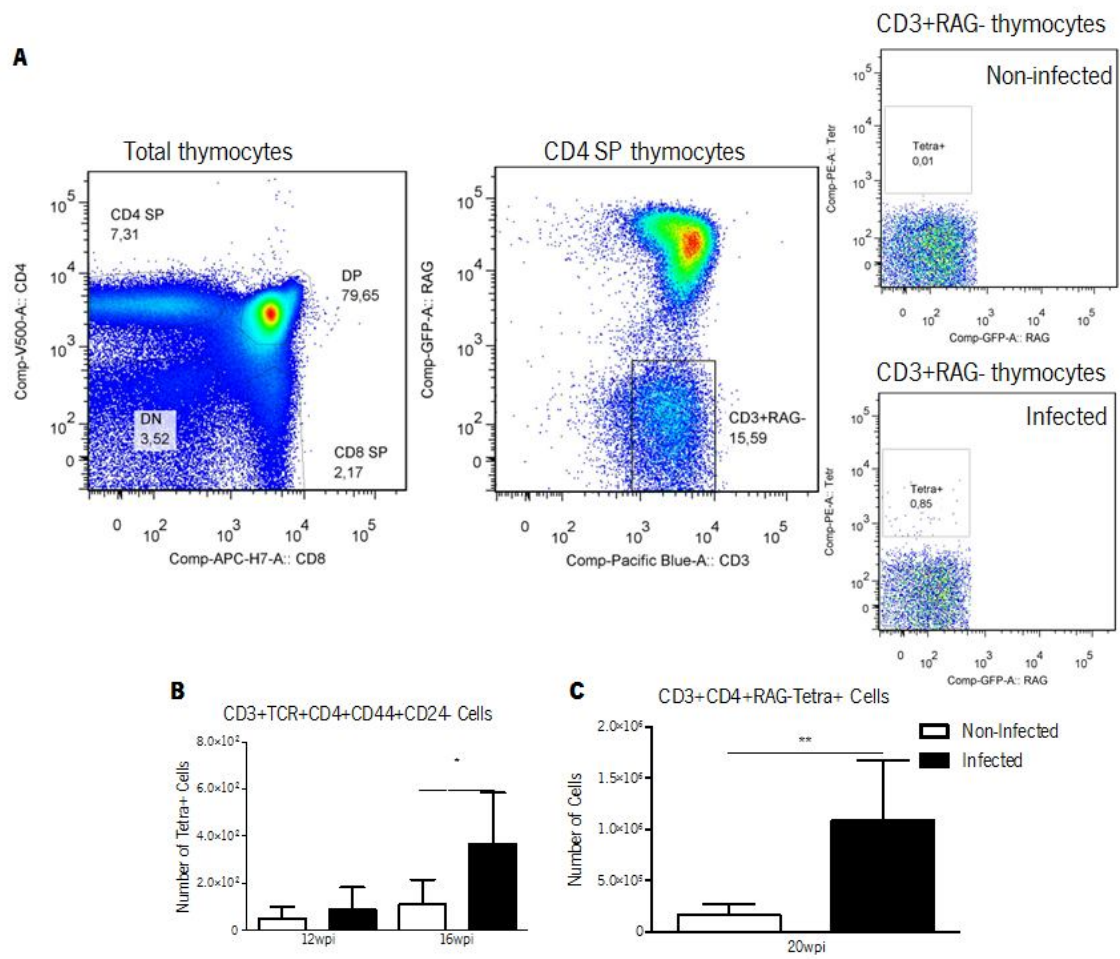
Figure 6: The pool of re-circulating T cells in the thymus is enriched with Ag85-specific T cells in late periods of infection. (A) Cytometry analysis of mycobacteria-specific re-circulating mature T cells in the thymus of RAG-GFP mice. (B) Re-circulating T cells from WT (CD44·CD24) or (C) RAG-GFP mice (GFP) are increased in Ag85 tetramer-positive cells at late stages of infection. Each column represents the mean and standard deviation of 6 to 9 mice per group, from one experiment. Significance was referred to as * when P≤0.05 and as ** when P≤0.005.

5. Newly differentiated or re-circulating T cells: which are the major producers of IFN-γ in thymic infection?

To understand which cells are involved in the thymic immune response, recently differentiated (GFP+) or peripheral re-circulating T cells (GFP), cells were sorted from the thymus of infected RAG-GFP mice, according to the expression of GFP, CD44 and CD24 (Fig.7B), and transferred to *M. avium* infected TCRα-/- mice (Fig.7A). Splenocytes were also transferred as positive (GFP) and negative (GFP+) controls, according to previous data from our lab (since T cells generated in an infected thymus have an impaired ability in responding to mycobacterial antigens) [137]. After 10 weeks of infection, TCRα-/- mice were sacrificed to access the level of protection conferred by each cell subset.

The following data is very preliminary, since the experiment has only been done a single time, and should be interpreted with caution. Animals receiving thymic GFP- cells (GPF- Thy), equivalent to re-circulating T cells from the periphery, seem to be enriched with mycobacterial Ag85-specific tetramer positive cells (Fig.7C). This pool of cells also seems more capable of producing IFN-γ, after stimulation with Ag85 (Fig.7D) but not with the combination of PMA+ION (Fig.7E). Even more interesting is the fact that this cell pool seems to be more enriched and active even though it is the one with the smaller number of cells (Fig.7F). Furthermore, the low T cell reconstitution may also be the cause for the lack of protection visible through CFU data from the liver (Fig.7G).
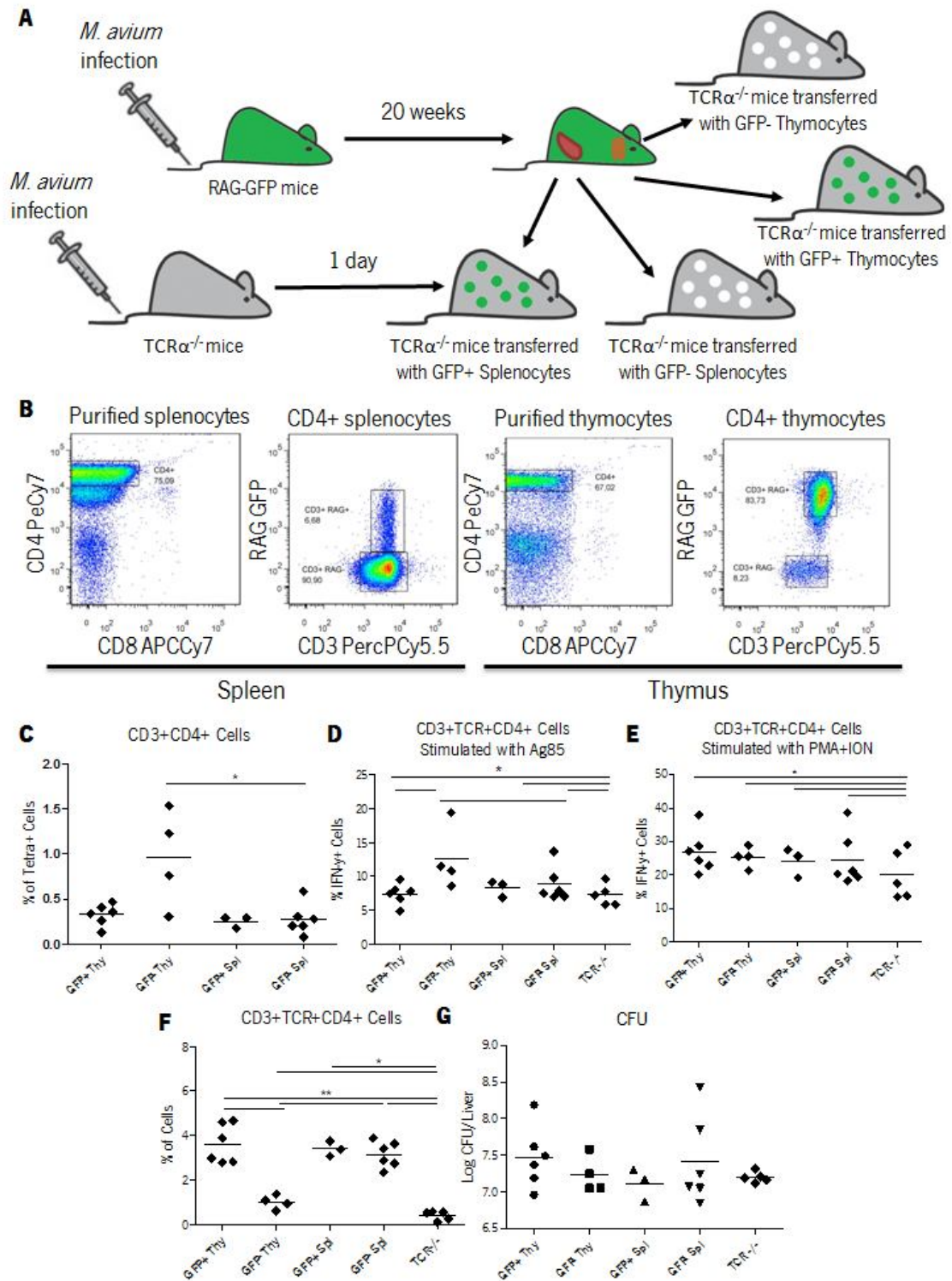
Figure 7: Mice that receive re-circulating T cells (GFP-) from RAG-GFP mice with 20 weeks of infection seem to be equipped with a more specific and activated pool of cells, despite having a lower T cell reconstitution. (A) After 20 weeks of infection, cells from thymi and spleens of RAG-GFP mice were sorted according to their expression of GFP, and CD44 and CD24 in the case of the thymus (B), and transferred to TCRα-/- mice. Animals were sacrificed after 10 weeks of infection. (C) GFP cells seem to be more enriched in Ag85 tetramer-positive cells, even with lower cell number. GFP cells stimulated with Ag85 also appear to produce more IFN-γ (D), but not when stimulated with PMA+ION (E). Although GFP- cells may come into view as a more specific and active pool of cells, no difference is observed in the CFU from the liver of these animals (G). Each column represents the mean and standard deviation of 3 to 5 mice per group. Significance was referred to as * when P≤0.05 and as ** when P≤0.005.

During systemic infection with *M. avium*, bacteria slowly but progressively, colonize the thymus, however after 15-17 weeks of infection, bacterial load is halted, entering into a stationary phase [22]. In peripheral organs, such as the spleen, this bacterial progression is also achieved, but in a shorter period of time. Bacterial progression in the spleen stops as early as 4wpi, and is associated with a peak of immune response mainly characterized by an increased in the production of IFN-γ, reaching the peak of expression at this time-point [26].

In accordance with what was known for the spleen, the bacterial plateau in the thymus is also attained when the production of IFN-γ reaches its higher expression. This elevated expression is coincident with the establishment of a plateau of bacterial load in the spleen at 4wpi, and in the thymus at 16wpi. The importance of this cytokine in mycobacterial infections has been widely demonstrated, especially in its ability of controlling bacterial growth [20, 21], and therefore our finding in respect to this cytokine in the thymus is in agreement to what was previously published for the spleen.

IFN-γ has a key role in activation of bactericidal mechanisms of APC, stimulating the expression of iNOS in infected macrophages. In the spleen, over-expression of IFN-γ is followed by iNOS production. However in the thymus, although IFN-γ is produced notably at 16wpi, iNOS was only detected at 24wpi. When looking at the immunofluorescence data, we can add that iNOS production is indeed clearly associated with infection, since its expression is associated in a great extent to infected cells. We have noticed, however that some infected cells do not present iNOS staining. Those cells usually present very few bacteria and tend to be isolated, while most infected cells tend to gather in clumps, with several bacteria and producing iNOS. It is possible that the delayed iNOS expression in comparison to IFN-γ expression may be due to an impaired cellular organization around infected cells within the thymus, since it has been shown that granuloma formation is directly influenced by proteins secreted by bacteria and that infected macrophages tend to cluster together [151].

Since, in the first weeks of infection, the bacterial load in the lung progresses in a very similar way to the one in the thymus, it would be interesting to evaluate the molecular similarities between both organs. In the lung, IFN-γ is expressed from as early as 4wpi, just as in the spleen. However, when comparing the IFN-γ gene expression it is far more up-regulated in the lung than the spleen, but with no visible effect in the bacterial load. Interestingly, although the vast up-regulation of IFN-γ, iNOS is only significantly increased after 24wpi. For this we speculate that T cells are not able to organize themselves in a correct manner to activate infected APC. The initial granuloma structures have been shown to expand bacterial numbers in zebrafish infected with *Mycobacterium marinum* [152]. This may furthermore help to explain the progressive bacterial growth in the lung. In addition high levels of IL-10 are produced in the lung from 12wpi, while in

the spleen the expression was transient. The elevated expression of this anti-inflammatory cytokine may also be delaying iNOS expression in the lung.

Extremely interesting is the fact that in the three organs studied, spleen, lung and thymus, at the time-point when iNOS starts to be significantly up-regulated in infected mice, the bacterial load was always above Log 5 CFU per organ. This may suggest that, in addition to the influence of IFN-γ and cellular organization [56], bacterial availability, or at least contact with bacilli, may aid in the creation of the correct environment for iNOS activation, as it was demonstrated in the case of *M. marinum* infected zebrafish [151].

One obvious observation is that the profile of immune activation in the thymus is delayed when compared to the spleen, but coincident with the cessation of bacterial progression. As the thymus has a very particular microenvironment, where cells and cytokines have different roles from those in other organs [138], we suspect that this environment may be influencing the way cells respond to infection. Although IFN-γ production is visible from the establishment of the bacterial plateau, its expression is not accompanied by iNOS, which appears later on during infection, neither by IL-10 to counter fight the pro-inflammatory cytokines.

Taking this into account, the immune activation in the thymus seems to be less intense when comparing to the one in the spleen and lung, with a rather moderate expression of IFN-γ and iNOS and no detectable up-regulation of IL-10. Interestingly, the thymus seems able to accommodate itself with the infection reaching bacteriostasis, like other organs, and not compromising T cell differentiation in general. In fact, it has been previously shown that infection with this strain of *M. avium* causes no significant atrophy in this organ and no significant alteration in the number of T cells that differentiate [137]. This work also adds that no alteration on the expression of cytokines, clearly involved in T cell differentiation, such as IL-7, is observed.

Antigen-specific IFN-γ producing T cells are known to be present, for example, as soon as 21 days after infection, in the lung of *M. tuberculosis* infected animals [35, 41]. As an essential molecule for combating intracellular bacteria, why is the IFN-γ profile and the bacterial plateau, in the thymus, established so far down the road after infection? Our laboratory showed previously that T cells exported by an infected thymus are unable to mount a protective immune response [137]. One could speculate that mycobacteria-specific T cells do differentiate but are retained within the thymus participating in the immune response within this organ. However, results published previously showed that for T cells to fully differentiate need to leave the thymus, enter circulation, and receive adequate stimuli in secondary lymphoid organs to fulfill this process [96-99]. However, this is only the case for adult RTE [90], since in newborns these cells are more capable of producing cytokines [89].

Taking all of this into account, we speculate that the cells participating in the thymic immune response are T cells which have fully differentiated in the periphery, and that are now re-circulating back to the thymus, as we will discuss bellow.

Being it extremely rare to find free bacteria in the blood, we suspect that DC homing to the thymus, loaded with bacteria, are involved in the slow colonization of the organ. DC have been shown to migrate into the thymus being able to participate in the differentiating process, either through clonal deletion of thymocytes [153] or by contributing to the induction of regulatory T cells [154]. Data from our laboratory show a slow increase of T cells specific for TB-10 (a peptide for *M. tuberculosis*) in *M. tuberculosis* infected thymi, while in the lung this increase is much more significant and from earlier time-points of infection (Nobrega and Nunes-Alves, unpublished data). This slow recruitment of mycobacteria-specific T cells might be following alongside the slow colonization of the thymus. As a complex organ, many may be the characteristics that influence bacterial progression, T cell recruitment and the immune response in the thymus (Fig.1).
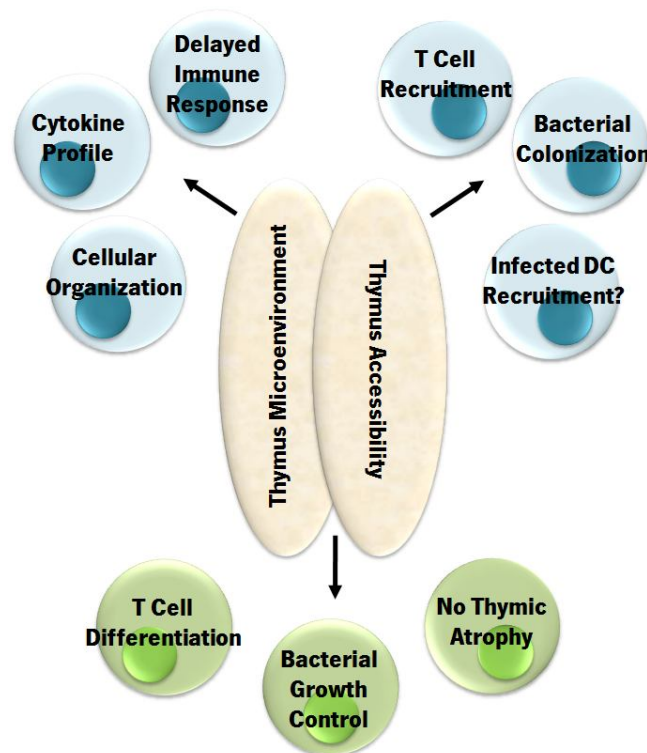
Figure 2: During infection with *M. avium*, the thymus is able to control bacterial proliferation while still maintaining its role in T cell education, with no visible signals of organ atrophy. The slow bacterial colonization of the organ is yet to be explained, however low bacterial load within the blood may suggest that these are being transported, for example, by dendritic cells (DC). As these cells progressively home to the thymus, T cells follow their path, in order to initiate the immune response. However, the thymic environment might not be so adequate for T cells to display all their characteristics. Consequently, the distinct profile of immunological activation together with the possible difficulty of cells to correctly arrange themselves in an organized structure, along with the slow colonization of bacteria and recruitment of T cells, may all be contributing to the delayed immune response and halting of bacterial progression within the thymus. Nevertheless, the thymus is able to control bacterial growth, with no significant signs of atrophy, and continue with its normal function of differentiating T cells.

Taking this into consideration, we started to analyse the involvement of T cells that were re-circulating, back to the thymus, from the periphery. Until very recently, no suitable markers have been described to characterize cells in re-circulation through the thymus of WT mice. However, in 2004, Boursalian *et al.* created a reporter mouse where newly generated T cells could be discriminated from the peripheral T cells that were re-circulating through the thymus with the aid of GFP expression during the somatic rearrangement of the TCR [96]. Using this model, in 2009,

Hale and Fink characterized re-circulating T cells (GFP) using cell surface markers by having high expression of CD44 and low expression of CD24 [88].

Using RAG-GFP mice, we compared the expression of CD44 and CD24 in GFP- cells and vice versa, to understand if these markers could be used to study re-circulation in WT animals. Indeed, about 80% of GFP- cells had a CD44+CD24- phenotype, while 90% of CD44+CD24- cells were GFP-, giving us a good approximation. Also, no significant difference was encountered when analysing cell number by both methods, giving us the reliability to use these markers to evaluate re-circulation in WT mice. However, since CD44 is a marker of cell activation, by excluding populations with lower expression of this marker, we may be excluding cells relevant for infection. Therefore, taking into account this limitation of using WT mice to evaluate re-circulation, data was also analysed using RAG-GFP mice.

We considered the possibility that, in infected mice, the number of re-circulating T cells would be increased. However, when analysing re-circulating T cells through the expression of CD44 and CD24, in the thymus of WT mice, or through the levels of GFP, in the thymus of RAG-GFP mice, no difference was observed between infected and non-infected mice at different periods of infection on the number of re-circulating T cells.

During infections by intracellular bacteria, Th1 cells express on their surface two major chemokine receptors, CCR5 (to which binds MIP-1β) and CXCR3 (to which binds IP-10 and MIG). Although the inflammatory chemokines IP-10, MIG and I-TAC, which all bind to CXCR3, have been shown to be expressed within the thymus, mainly to respectively participate in the differentiation process of CD8SP, $\gamma\partial$T cells and Natural Killer (NK) T cells [155], MIP-1β has not. So, the combination of the three different chemokines that bind to two different receptors present on Th1 cells, poses a more correct way to analyze the recruitment of these cells to the thymus. When analyzing organs for the expression of these chemokines, an increased production was observed in the spleen and lung from 4wpi, and from 16wpi in the case of IP-10 and MIG in the thymus. So maybe not overall re-circulating of T cells is increased to the thymus, but only recruitment of mycobacteria-specific Th1 cells.

To further analyse the specificity of re-circulating cells, specific tetramers for mycobacterial antigens were used. When analyzing the specificity of re-circulating T cells, we saw that this pool was enriched with mycobacteria-specific T cells on WT mice (CD44+CD24-cells), after 16wpi, and on RAG-GFP mice (GFP-cells), at 20wpi. This might suggest that the thymus is recruiting specific T cells, which will be responsible for the bacteriostasis.

To understand if re-circulating T cells are active producers of IFN-γ, after 20 weeks of infection, thymocytes from RAG-GFP mice were sorted according to the expression of GFP and transferred into *M. avium* infected TCR-/- mice (mice lacking αβT cells). As controls, splenocytes were also sorted and transferred according to GFP expression. GFP+ spleen cells were used as a negative control, since our lab has shown that T cells originated from an infected thymus have an impaired ability in responding to mycobacterial antigens [137]. GFP- spleen cells were used as a positive control, since these cells are a representation of the peripheral pool of T cells, specific for

several antigens, including mycobacterial antigens. After 10wpi we have assessed the specificity of each cell subset and also their ability to produce IFN-γ.

Animals receiving thymic re-circulating T cells (GFP), seem to have a pool of cells more enriched in mycobacteria-specific T cells, than those that received newly differentiated cells (GFP+). Furthermore, these cells are more prone to produce IFN-γ when stimulated *in vitro* with Ag85. All cells respond equally to the mitogens PMA+ION, which indicate that cells have no difficulty in producing IFN-γ. The number of transferred cells merely allowed the characterization of each cell subset. Low T cell number in the spleen may also be due to the migration of these cells directly to the thymus of the receptor mouse upon transfer, since this is the environment where they previously were. This experiment has not yet been repeated, and therefore, data is extremely preliminary. To improve the readout of this experiment, also allowing the assessment of protection induced by each cell subset, this shall be repeated, however with less receptor mice, in order to increase the number of transferred cells per receptor.

With the data presented in this work, it seems correct to assume that the thymus not only is able to mount an immune response, but seems able to assemble a very specific response, without extremely elevated levels of pro-inflammatory molecules, and therefore preserving both physical and functional integrity. Furthermore, our results strongly suggest the role of thymic immune surveillance to re-circulating T cells in the thymus.

In this work we have been able to show that the thymus is a site for an immune response, able to mount a profile of immune activation which is distinct and more delayed when compared with those in the spleen and lung. Levels of IFN-γ in this organ are elevated at the same time point that bacterial growth is stabilized. Infection also leads to an elevated expression of chemokines, indicating the recruitment of Th1 cells. Since newly differentiated T cells have not fulfilled their maturation process until they leave the thymus, we suspected the involvement of re-circulating cells in the control of the infection in this organ. Even though re-circulating cells are not increased in number, they are enriched with mycobacterial-specific T cells. Preliminary data from our laboratory also indicate that these re-circulating cells seem to be more active producers of IFN-γ, in comparison with newly differentiated T cells.

The literature has shown that after leaving the thymus and fully maturating in the periphery, T cells are able to return to this organ. However, no explanation has been giving for why these cells do so. Consequently, this work suggests a role for re-circulation: cells that re-circulate back to the thymus are part of a simple surveillance routine.

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