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Trabalho Efetuado sob a orientação do
Doutor António Gil Castro
e da
Doutora Lúcia Moreira Teixeira

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DECLARAÇÃO

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Para ser grande, sê inteiro: nada teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és no mínimo que fazes.

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ABSTRACT

The host immune system needs to respond to the pathogens with adequate intensity and duration to control and eradicate the infection, without compromise the host homeostasis. In this respect, several mechanisms are involved in the control and regulation of the immune response to prevent an exaggerated immune response to pathogens. Here, we focused on two key molecules and addressed their impact in the control of the intracellular pathogens *Mycobacterium tuberculosis* and *Listeria monocytogenes*: interleukin (IL)-10, an important anti-inflammatory cytokine; and sirtuin2 (SIRT2), a histone deacetylase with important anti-inflammatory properties poorly explored during infection.

To address the role of IL-10 during different phases of the infection, we used the pMT-10 mice model, which over-expression of IL-10 is under the control of a zinc-inducible promoter.

We show that IL-10 over-expression during early and chronic phase of immune response to *M. tuberculosis* did not significantly impact the immune response. When IL-10 was over-expressed during the period corresponding to T cell priming, the immune response was altered but without consequences in the control of infection. Specifically, we observe an increase in interferon-γ-producing CD4 T cells and a decrease in IL-17-producing CD4 T cells. The myeloid response was also altered, with DC expressing low MHC class II and, surprisingly, inflammatory monocytes expressing more MHC class II. On the other hand, in *L. monocytogenes* infection high levels of IL-10 increased susceptibility to infection. In sheer contrast with *M. tuberculosis* infection, the susceptibility of pMT-10 mice to *L. monocytogenes* was associated with a significant decrease in number and activation of inflammatory monocytes in the spleen.

In what regards SIRT2, we observed that this deacetylase did not significantly impact the inflammatory response of macrophages *in vitro* or *in vivo* the control of *M. tuberculosis* in the lung. Interestingly however, we found higher bacterial burdens in the liver of SIRT2 deficient mice, suggesting that this deacetylase may have an important impact in the dissemination of mycobacteria.

Understanding the temporal and site-specific impact of immunomodulatory pathways during infection will be a significant step towards understanding the dynamics between the pathogen and host immune response. This will have an important impact in the development of more rational therapeutic approaches.

RESUMO

A intensidade e duração da resposta imune são essenciais para garantir o controlo de agentes infeciosos sem comprometer a homeostasia do hospedeiro. Diferentes mecanismos, cujo papel é a regulação da intensidade da resposta imune, foram já identificados como sendo importantes para prevenir respostas exageradas do hospedeiro. Nesta tese, estudamos o papel de duas moléculas, a interleucina-10 (IL-10) e a sirtuina 2 (SIRT2), uma citocina e uma histona deacetilase com importantes propriedades imunossupressoras e anti-inflamatórias, na infeção por *Mycobacterium tuberculosis* e por *Listeria monocytogenes.*

Para avaliar o papel da IL-10 durante as diferentes fases da infeção por *M. tuberculosis*, utilizamos ratinhos transgénicos (pMT-10) que sobre-expressam IL-10 sobre controlo de um promotor induzido por zinco. A sobre-expressão de IL-10 durante as fases inata e crónica da resposta imune não influenciou a capacidade dos ratinhos para controlar a infeção por *M. tuberculosis,* enquanto que a sobre-expressão da IL-10 no período de tempo correspondente à ativação das células T levou a alterações da resposta imune do hospedeiro, sem alterar o controlo da infeção. Nesta fase da infeção, após a indução de níveis elevados de IL-10, observou-se um aumento nas células CD4 produtoras de interferão (IFN)-γ e uma diminuição das células CD4 produtoras de IL-17, nos pulmões. A indução de níveis elevados de IL-10 também influenciou a ativação das células mieloides, observando-se uma menor expressão de MHC-II pelas células dendríticas mas curiosamente uma maior expressão de MHC-II pelos monócitos inflamatórios.

Por outro lado, na infeção por *L. monocytogenes*, a indução de níveis elevados de IL-10 aumentou a suscetibilidade dos ratinhos pMT-10 à infeção, que poderá estar relacionado com a diminuição do número de monócitos inflamatórios e da sua menor ativação no baço.

Relativamente ao papel da SIRT2, observamos que a ausência deste mediador nas células mieloides não influenciou a resposta dos macrófagos nem o controlo da infeção *in vitro* por *M. tuberculosis.* Curiosamente, *in vivo* observamos um aumento da carga bacteriana no fígado, o que sugere que esta deacetilase pode ter um papel importante na disseminação desta micobactéria.

A compreensão das vias imunomoduladoras durante a infeção será um passo importante para a compreensão da dinâmica entre o agente patogénico e a resposta imune do hospedeiro. Isto poderá ter um impacto importante no desenvolvimento de abordagens terapêuticas mais racionais.

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APC	Antigen presenting cell
BCG	Bacillus Calmette-Guérin
BMDM	Bone marrow-derived macrophages
BHI	Brain heart infusion
CCL	CC-chemokine ligand
CCR	Chemokine receptor
CLR	C-type lectin receptor
CXCL	chemokine (C–X–C motif) ligand
CR	Complement receptor
DCs	Dendritic cells
CFU	Colony-forming unit
FBS	Fetal bovine serum
FoxP3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDAC	Histone deacetylase
HAT	Histone acetyltransferases
HIV	Human immunodeficiency virus
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I.P	Intraperitoneal
IRF	Interferon regulatory factors
LLO	Listeriolysin O
MAPK	Mitogen activated protein kinases
MARCH1	Membrane associated ring-CH1
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary-response protein 88
NAD	Nicotinamide adenine dinucleotide

NF-kβ	Nuclear factor kappa beta
NK	Natural killer
ON	Overnight
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PRR	Pattern recognition receptors
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RT	Room temperature
Sir2p	Silent information regulator 2 protein
SIRT	Sirtuin
TB	Tuberculosis
Th	T helper
TLR	Toll like receptor
TGF-β	Transforming growth factor
TNF	Tumor necrosis factor
Treg	T regulatory
TRIF	TIR domain-containing adaptor inducing interferon
Zn	Zinc

INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system, composed by two functional subsystems, the innate and the adaptive immune systems, that have evolved, to protect the host from infectious agents that exist in the environment, by reciprocal selective pressures and diverse invasion and evasion mechanisms on both participants [1, 2]. The innate immune system confers the first line of defense to infection, it is crucial for the early pathogen recognition which has a critical impact in the development of the protective response [1, 2]. On the other hand, the adaptive immune system triggers a specific response often able to clear the pathogen and to generate immunological memory [2].

1.1.1 Innate Immunity

The innate immune response is orchestrated at different levels. It is composed by the physical barriers, as epidermis, ciliated epithelium, endothelium and mucosal surfaces that produce antimicrobial secretions, which prevent the entry and spread of microorganisms [1, 2]. Wherever physical barriers are unable to prevent infections, the innate immune response takes over. This response encompasses chemical components such as soluble receptors, complement proteins and enzymes that hydrolyze the pathogen; and cytokines and chemokines that drive the immune response [2-4]. The cellular components of the innate immune system includes phagocytic cells (macrophages, neutrophils, monocytes and dendritic cells (DCs)), innate lymphoid cells (natural killer (NK) cells, NKT cells and γδ T cells), mast cells, basophils and eosinophils that are involved in the recognition of pathogens and in the triggering of the antimicrobial immune response [2-4].

The innate immune response starts with the recognition of evolutionarily conserved structures of pathogens, known as pathogen-associated molecular patterns (PAMPs) through a limited number of germ line-encoded pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), c-type lectins receptors (CLRs) and others; and also complement receptors (CR) [2, 5-7]. Upon PAMPs recognition, different intracellular signalling pathways downstream PRRs can be triggered. These pathways are mediated by adaptor molecules, including the myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF). The main signalling pathways downstream TLR-mediated responses are nuclear factor kappa B (NF-kβ) [2, 7-9] and mitogen-activated protein kinases (MAPKs) [7, 10]. These cascades are the principal responsible for the induction of IL-10 and pro-inflammatory cytokines and, also interferon regulator factors (IRFs) [7] that are crucial for the production of type I IFN [2, 7]. In

addition to these antimicrobial responses, the innate immune system has other mechanisms that recognize and limit the growth of pathogens such as, the complement system activation, phagocytosis by macrophages and polymorphonuclear cells, and autophagy [1, 4]. Furthermore, the innate immune system is also able to recognize and clear host factors that appear in abnormal locations or in aberrant conformations such as, extracellular DNA or ATP resulting from cell death caused by infection or stress [11].

1.1.2 Adaptive Immunity

The adaptive immune response is initiated with the presentation of antigens to naive T cells by antigen-presenting cells (APCs), including DCs, macrophages and B cells [12, 13]. The generation of antigen-specific responses begins with the recognition and internalization of pathogens, or their antigens, normally by DCs in peripheral tissues. These cells then migrate to the draining lymph nodes where antigens are presented to naive T cells in the context of major histocompatibility complex (MHC) molecules [2, 12, 14]. Pathogen recognition by PRRs has a critical role in this process as it promotes DC maturation and enhances their migratory phenotype [15]. This phenotype is characterized by the upregulation of MHC class II, costimulatory molecules (such as CD40, CD80 and CD86), cytokines (such as interleukin (IL)-12, IL-6 and tumor necrosis factor (TNF)) [14, 16, 17], and lymphoid tissue homing chemokine receptors (such as CCR7) [18, 19]. Altogether, the recognition of the complex MHC-peptide by T cells, together with costimulatory signals and the cytokine microenvironment promote the differentiation of naive T cells towards specific populations [13].

DCs are able to induce the differentiation of naive CD4 T cells into different helper (Th) subsets, including Th1, Th2 and Th17 characterized by a specific cytokine signature [20-23]. This differentiation is initiated by PAMP recognition by the APC that ultimately determines the type of cytokines produced [21]. In this regard, IL-12 produced by APCs [24], together with IFN-γ from memory T cells and NK cells, plays a critical role in inducing the Th1 phenotype [20, 21, 25]. IL-4 is in turn involved in the induction of Th2 responses [20, 21, 25, 26]. Finally, IL-1, transforming growth factor (TGF)-β and IL-6 promote the differentiation of Th17 responses and IL-23 maintains their phenotype [27-30]. The Th1 subset, characterized by the expression of IFN-γ, IL-2, and TNF, is critical for protection against intracellular pathogens by promoting macrophage activation [20-22]. Th2 cells, essential in immunity to extracellular pathogens and involved in allergic responses, are characterized by the production of IL-4, IL-5, IL-13 and B cell growth and

differentiation factors that support humoral immunity [20-22]. Th17 cells, important in protection against several pathogens, including intracellular and extracellular pathogens, are characterized by IL-17, IL-21 and IL-22 production [22, 27]. The activity of these effector cells is strictly regulated between them [31] and by natural and inducible regulatory T (Tregs) cells, whose differentiation and maintenance are dependent on the transcription factor Foxp3 [23, 32]. An abnormal activity of Th1 or Th17 cell responses may culminate in autoimmune diseases [22, 33], whereas excessive Th2 cell responses may result in allergic inflammatory disease [22]. In all, the innate and adaptive immune responses are interactive subsystems of the immune system that act in concern to protect the host from infection but can also cause important immunopathological consequences. Therefore, it is essential to understand how the balance between pathogen clearance and immunopathology in several infections is regulated.

1.2 THE IMMUNE RESPONSE TO INTRACELLULAR BACTERIA

Intracellular pathogens have the capacity to enter and to explore survival mechanisms inside the cells. As a result, the host immune protective response is mainly mediated by T cells with little participation of the humoral response [34]. Furthermore, the response to intracellular bacteria culminates in tissue reactions typically characterized by granuloma structures [34]. Despite intracellular bacteria being contained within these granulomata, sometimes pathogen clearance fails and the disease becomes chronic. Two classical examples of intracellular pathogens are *Mycobacterium tuberculosis* and *Listeria monocytogenes.*

1.2.1 M. tuberculosis Infection

Tuberculosis (TB) is an infectious disease caused by the bacillus *M. tuberculosis*, which is spread by respiratory transmission [35]. Typically, it affects the lungs (pulmonary TB), however this disease can be extrapulmonary [35, 36]. TB was declared by the World Health Organization (WHO) a global public health emergency in 1993 [35]. Despite the many efforts to improve TB prevention and treatment, TB remains a major cause of morbidity and mortality worldwide [35]. There were 8.6 million new cases and 1.3 million deaths in 2013 and it is estimated that one-third of the world's population is currently infected with *M. tuberculosis* but remain asymptomatic, defined as latent TB [35]. Only 5-10% of these latent individuals develop active TB disease in their lifetime [37, 38]. Control of this global epidemic disease has been impaired by the lack of an effective vaccine, as the only available vaccine, *Mycobacterium bovis* bacillus

Calmette-Guérin (BCG), is only efficient against childhood TB [39-41]; by the appearance of multidrug-resistant *M. tuberculosis* strains [42, 43]; by co-infections with the human immunodeficiency virus (HIV) [44, 45]; and by the lack of sensitive and rapid TB diagnosis [46].

1.2.1.1 Innate immune response

Once the bacillus is delivered to the lungs, the immune response to *M. tuberculosis* is initiated with the bacterial recognition by phagocytic cells, mainly alveolar macrophages but also neutrophils, monocytes and DCs [47-51], through different PRRs expressed on the surface of these cells [52-54]. Among TLRs, TLR2, -4 and -9 are the best characterized in the recognition of *M. tuberculosis* [54-56]. Briefly, TLR2 recognizes the largest array of mycobacterial PAMPs, including lipoproteins, phosphatidylinositol mannans and lipomannan; TLR4 recognizes phosphatidyl-myo-inositol mannosides (PIMs) [56, 57], and TLR9 senses mycobacterial DNA [55, 57]. *M. tuberculosis* recognition via different TLRs triggers various intracellular signaling cascades that culminate with the production of important pro- and anti-inflammatory cytokines involved in protective host immune response, including TNF, IL-12 (both pro-inflammatory) and IL-10 (anti-inflammatory). There is experimental evidence supporting the key role of multiple TLRs in the control of *M. tuberculosis* [58-61]. Moreover, different polymorphisms in the human TLR2 gene were reported to associate with increased susceptibility to TB [62-64].

Upon *M. tuberculosis* recognition, phagocytic cells upregulate different microbicidal mechanisms. Alveolar macrophages are the primary cell type involved in the uptake of mycobacteria [50, 65, 66] and the depletion of these activated cells in mice led to impaired resistance to infection [67]. *In vivo* and *in vitro* studies in mice have shown that *M. tuberculosis* is phagocyted mainly by alveolar macrophages [68] but DCs [69, 70] and neutrophils [71-74] can also phagocytose *M. tuberculosis*. These cells protect the host in a non specific manner through essential microbicidal mechanisms, such as the production of toxic effector molecules such as reactive oxygen or nitrogen intermediates (ROI or RNI, respectively), phagossome acidification, limiting iron availability and undergoing apoptosis [47, 50, 51, 75]. Indeed, mice lacking the mediators of microbicidal macrophage functions, such as expression of inducible nitric oxide synthase (iNOS) [48, 76] or LRG-47 [77] are unable to control *M. tuberculosis* infection.

1.2.1.2 Adaptive immune response

Th1 response

The innate immune response makes a critical contribution to the activation of the adaptive immunity. Indeed, inflammatory response mediated by phagocytic cells increase the flow of DCs, which around day 9 post-infection start to migrate to the draining lymph nodes, where they will drive naive T cell activation and differentiation (Figure 1) [78-81]. Some reports have shown that *M. tuberculosis* compromise DCs function, which may explain the slow dissemination of bacteria to the lymph nodes [69, 82, 83]. Upon T cells activation, after 2 weeks of infection, they begin migrating to the lung where they mediate protective immunity by activating the infected phagocytes (Figure 1) [81, 84].

It is well established that CD4 T cells capable of making IFN-γ are required for protective immunity [85, 86] and that loss of these cells increases the probability of mice to succumb to TB [44]. Indeed, CD4 T cells-producing IFN-γ (Th1) are required for resistance to lethal infection, given that MHC class II [87] and IFN-γ [88-90] deficient mice rapidly succumb to infection. IFN-γ deficient mice fail to produce RNI and ROI and develop progressive tissue destruction, associated with uncontrolled bacterial growth [90]. These data highlight the important role of IFN-γ in the control of *M. tuberculosis* infection; however, recent studies suggest IFN-γ-independent mechanisms whereby CD4 T cells mediate protection [91]. Although antigen specific CD8 T cells, NK cells and γδ T cells also produce IFN-γ during *M. tuberculosis* infection, they cannot compensate for a lack of CD4 T cells [80, 81].

The induction of protective IFN-γ T cell response depends upon IL-12 [92, 93] which is mainly produced by activated DCs. Indeed, IL-12p40 deficient mice cannot control bacterial growth and this is associated with the absence the innate and adaptive sources of IFN-γ [92, 94]. Recently, IL-12p40 has been suggested to induce DCs migration to the lymph nodes [95], thus promoting T cell priming [95, 96]. Human studies have also shown a crucial role for the IL-12-CD4-IFN-γ axis in the control of mycobacterial infections [97]. Indeed, humans with mutations in gene encoding the IL-12 receptor are extremely susceptible to *M. tuberculosis.* Similarly, patients with mutations in IFN-γ receptor 1 (IFN-γR1) and in gene encoding STAT1, an essential transducer of IFN-γ-mediated signals, are also extremely susceptible to pulmonary TB and BCG infection [97, 98]. Also in humans, CD4 T cells are critical in the control of *M. tuberculosis* as reported by the increased susceptibility of humans infected with the HIV [44].

In summary, the axis IL-12-CD4-IFN-γ is critical to the control of *M. tuberculosis* infection, although these cells are unable to eradicate the pathogen or to prevent the chronic disease. Therefore, it is important to understand as the protective immune response to *M. tuberculosis* is regulated, for instance, by IL-10, in order to design novel immune therapies.

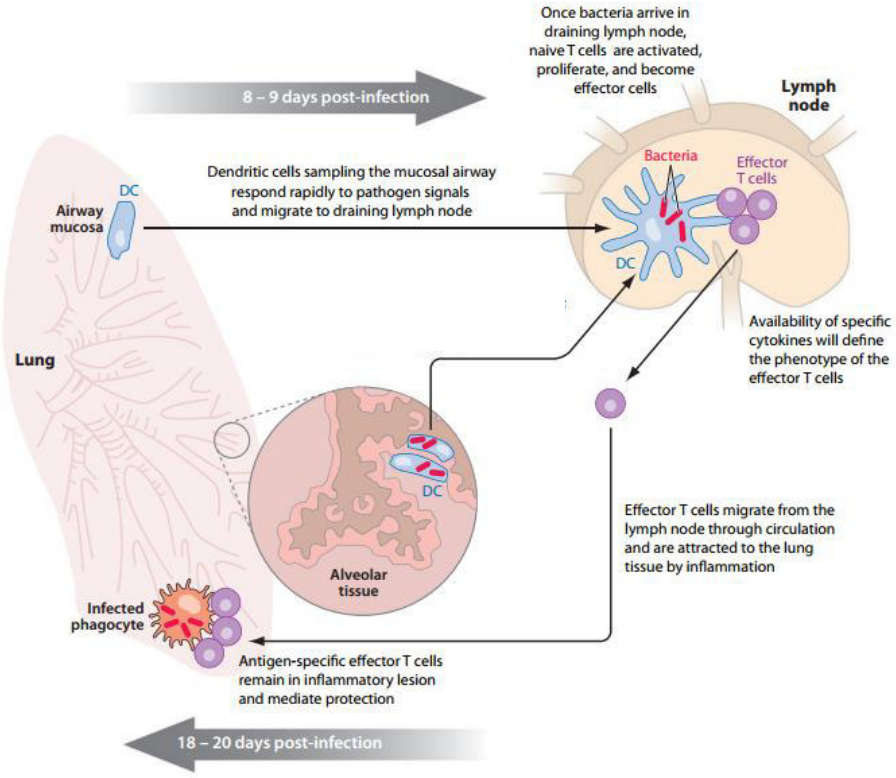

Figure 1. Cell-mediated immune response in *M. tuberculosis* infection. Upon *M. tuberculosis* aerosol infection, the bacillus is deposited in the lower airways and the alveolar tissue. *M. tuberculosis* is recognized by alveolar macrophages and DCs and the bacteria do not disseminate from the lung until day 9 post-infection. After, DCs migrate to the lymph nodes and present the antigen to naive T cells. The phenotype of T cells will depend on the availability of specific cytokines. After T cell activation and differentiation, these cells migrate to the lung in response to inflammation and mediate protection by macrophages further activation, stopping the bacterial growth. From Andrea M. Cooper, Cell-mediated Immune Response in Tuberculosis, Annual Reviews of Immunology, 2009

Th17 response

In addition to Th1 cells, Th17 cells are also involved in the immune response to mycobacteria [99, 100]. However, in the absence of IL-17, protection is not altered in response to a low dose of *M. tuberculosis* infection [99]. On the other hand, when IL-17 is blocked during a high-dose challenge, the recruitment of neutrophils, granuloma formation and protection are impaired [101, 102]. In the context of vaccination, repeated exposure to BCG results in a significant increase in IL-17 response, accompanied with an increase in influx of neutrophils/granulocytes and subsequently, pathological inflammation in the lungs [103]. Furthermore, Th17 cells seem to be able to anticipate the recruitment of Th1 cells to the lung, promoting the resistance to mycobacterial infection [101]. It has also been shown that IFN-γ can inhibit CD4 T cell-producing IL-17, impairing the recruitment and the accumulation of neutrophils, contributing to decreased lung inflammation and improved the outcome of the disease [104]. Thus, the IL-17 cellular response needs to be tightly regulated because the balance between protective and damaging immunity need to be achieved to control of *M. tuberculosis* proliferation and reduce transmission and mortality.

1.2.2 L. monocytogenes Infection

L. monocytogenes is the causative agent of human listeriosis, a potentially fatal foodborne infection [105]. The incidence of listeriosis varies between 0.1 and 11.3/1000000 in different countries [106]. Despite adequate antimicrobial treatment, listeriosis has an average case-fatality rate of 20-30% [106]. Pregnant woman and individuals who are immunocompromised or elderly are particularly vulnerable to *L. monocytogenes* infection [107]. Clinical manifestations are variable, ranging from febrile, gastroenteritis to more severe invasive forms, including sepsis, meningitis, rhombencephalitis, perinatal infections, and abortions [105, 107]. Contrary to *M. tuberculosis*, these bacteria cause an acute infection and the major targets organs of experimental murine listeriosis are the spleen and liver [34] The murine model of *L. monocytogenes* infection is an attractive tool to understand the interplay between the host and pathogen [108]. Infection of mice with a sub-lethal dose of *L. monocytogenes* induces a robust innate inflammatory response, that restricts bacterial growth and dissemination to the liver and spleen, preventing the spread into systemic and, consequently lethal infection [108].

1.2.2.1 Innate Immune Response

Upon intravenous infection of mice, the bacteria are rapidly taken up by resident myeloid cells of spleen, including macrophages, neutrophils and DCs [108, 109]. Within the cell, *L. monocytogenes* escapes the phagosome to cytosol by producing listeriolysin O (LLO), a virulence factor that destroys the phagosomal membrane (Figure 2a) [110, 111]. In the cytosol, *L. monocytogenes* induce NF-kβ pathway mediating the transcription of innate inflammatory cytokines and chemokines, as CC-chemokine ligand 2 (CCL2) and CCL7 (Figure 2b) [107]. These chemokines are involved in the recruitment of $Ly6C^{High}$ inflammatory monocytes to infected tissues [107, 109]. The recruitment of these inflammatory cells is essential for the control of *L. monocytogenes* infection [112]. In response to microbial products, macrophages secrete TNF and IL-12 (Figure 2c). These cytokines drive NK cells to produce IFN-γ, which in turn increases the antimicrobial capacity of macrophages.

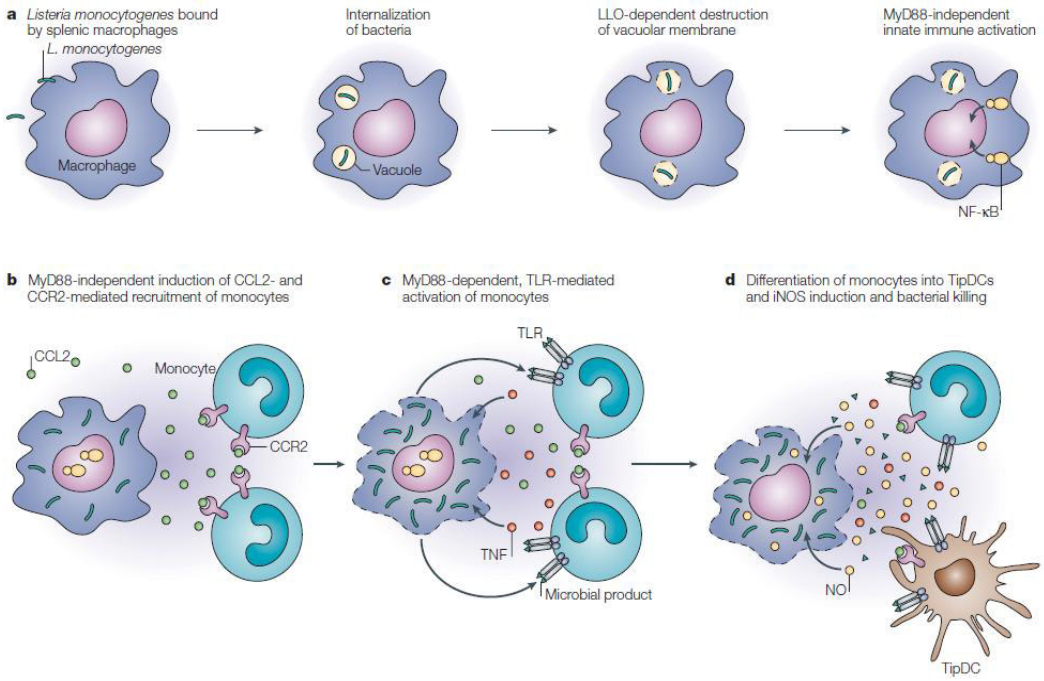

Figure 2. Innate immune activation by *L. monocytogenes*. (a) *L. monocytogenes* in the bloodstream is rapidly recognized and internalized by macrophages in the spleen and other tissues. In the macrophages phagossome, bacteria secrete LLO, which lyses the phagossome membrane and activates NF-kβ pathway, mediating the transcription of innate immune genes, as CCL2. **(b)** CCL2-producing infected cells induce the recruitment of circulating monocytes that express CCR2, to the infected tissues. **(c)** Infected macrophages release microbial products that activate recruited monocytes through TLRs in MyD88-dependent manner. **(d)** Monocytes differentiate in DCs that release TNF and nitric oxide (Tip-DCs), which promote bacterial killing. From Eric G. Pamer, Immune Responses to *Listeria monocytogenes*, Nature Reviews Immunology, 2004

Accordingly, IFN-γ and TNF deficient mice are highly susceptible to *L. monocytogenes* infection [108, 109, 113]. Thus, the innate immune response is essential for host survival, as indicated by the impact in the outcome of infection on mice that lack the innate immune players [113]. Despite the importance of innate immunity for the initial control of *L. monocytogenes*, the T cell response is needed to bacteria clearance since SCID mice develop chronic infection [114].

1.2.2.2 Adaptive Immune Response

DCs play a central role in the recognition of *L. monocytogenes,* antigen presentation and subsequent priming of T cells [107, 108]. Indeed, mice depleted of CD11c+ DCs are unable to generate a protective CD8 T cell response [115]. In this regard, CD8 T cells provide a more substantial contribution to a long-term protective response than CD4 T cells to *L. monocytogenes*. These cells can be separated into two populations: the first is restricted to classical MHC class I molecules, and the second is restricted to a non-classical MHC class I molecule (H2-M3-restricted T cells) [107]. During primary infection, H2-M3-restricted CD8 T cells respond rapidly, whereas MHC class I-restricted CD8 T cells reach peaking frequencies only 7 to days following inoculation [107]. During a recall response however, MHC class I- restricted CD8 T cells are more important [107]. CD8 T cell response contributes to anti-listerial immunity by two synergistic mechanisms: lysing infected cells via perforin and granzymes, exposing intracellular bacteria; and secreting IFN-γ to activate macrophages [116].

The role of CD4 T cells during *L. monocytogenes* infection is still not well understood, but it is thought that CD4 T cells differentiate into Th1 cells [117] and facilitate granuloma formation [118]. Furthermore, CD4 T cells were shown to contribute to CD8 T cells activation, but mice lacking CD4 T cell responses mount a primary CD8 response similar to wild-type mice and rapidly clear the infection [108, 119]. In addition, *in vivo* depletion studies shown that memory CD8 T cells are the most effective T cell subset able to confer protection [120].

In all, murine *L. monocytogenes* infection provides an interesting model to address innate and acquired mechanisms of protection during infection, including the role of cytokines and their function in different cell lineages.

1.3 MODULATION OF THE IMMUNE RESPONSE

During infection, the host immune system needs to respond to pathogens with adequate intensity and duration to control and eliminate the infection. However, exacerbated antimicrobial mechanisms can often cause collateral damage, which sometimes is more detrimental to the host than the infection itself. Thus, several mechanisms have been described to control and regulate the immune response to prevent immunopathological consequences. In the next section, the importance of two key molecules in the inflammatory response, IL-10 – an important mediator in immune response against several pathogens, and sirtuin2 – an immune mediator unexplored in the context of *M. tuberculosis* infection are discussed.

1.3.1 IL-10 – an immunosuppressive cytokine

IL-10 Biology

IL-10 is an anti-inflammatory cytokine produced and recognized by many immune cells, from both myeloid and lymphoid lineages [121, 122]. Among innate immune cells, monocytes, macrophages and DCs (but not plasmocytoid DCs [123]) are the main producers of IL-10, but NK cells, mast cells and granulocyte cells are also able to synthesize this cytokine [121, 122]. In addition, many subsets of T cells can produce IL-10, such as Th1 cells, Th2 cells, CD8 T cells, and Treg cells; and also B cells. Recently, Th17 and Th22 were also found to produce IL-10 [121, 122].

IL-10 acts through a surface receptor complex – IL-10R, which is composed by IL-10R1 and IL-10R2 subunits [121, 122]. This receptor transduces signals through the JAK-STAT signalling pathway and STAT transcription factors [122, 123]. IL-10 production is induced in response to the interaction between TLRs and PAMPs and it may also be induced through CLR, Card9 and DC-SIGN [122-124]. Moreover, IL-10 can be induced by pathogens as an evasion mechanism [125]. However, it is still not clear whether high concentrations of IL-10 during infections are a cause or a consequence of high pathogen loads [124].

The immunosuppressive action of IL-10 has a broad range of target cells in an autocrine/paracrine way, mainly on the innate cells, namely on macrophages and DCs. IL-10 produced early in the immune response can inhibit the release of pro-inflammatory mediators (TNF-α, IL-1β , IL-6, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF) and others) by macrophages [121, 126, 127], as well as the expression of chemokines involved in

the recruitment of monocytes, DCs, neutrophils and T cells [121, 122, 124]; inhibit phagocytosis and suppress the production of ROI and RNI, such as nitric oxide, involved in bacterial killing [121, 125, 128]. In addition, IL-10 blocks antigen presentation by APCs via down-regulation of MHC class II and co-stimulating molecules (e.g. CD86 and CD80) [121, 122]. Moreover, IL-10 inhibits APCs migration to the lymph nodes by dampening IL-12 production [124]. As result, the effects of IL-10 in DCs and macrophages indirectly compromise the activation and differentiation of naive T cells and, subsequently, adaptive immunity [121, 129]. Moreover, the effect of IL-10 in Th1 cells decreases IFN-γ production and, consequently, amplifies the deactivation of APCs [121]. IL-10 also reduces IL-23 production by macrophages, which is essential for the proliferation and survival of Th17 cells [122]. In addition, IL-10 can also inhibit T cells proliferation and differentiation by impairing the production of cytokines important for these effects, namely, IFN-γ, IL-2, IL-4 and IL-5 [121, 124, 127].

Despite the inhibitory functions of IL-10, this cytokine is also important in the prevention of apoptosis of B cells, and in the recruitment, proliferation and cytotoxic activity of CD8 T cells and NK cells [121, 127]. Thus, IL-10 can directly regulate innate and adaptive responses by limiting T cell activation and differentiation in the lymph nodes, as well as suppressing proinflammatory responses in tissues, leading to impaired control of infections and/or reduced immunopathology.

IL-10 in Infections

Depending on the type of infection, the presence of IL-10 may either contribute for host protection or susceptibility [121, 127]. In certain cases, the presence of IL-10 prevents exacerbated inflammatory responses that frequently culminate in fatal immunopathology, such as those occurring upon infection with *Toxoplasma gondii* [130], *Plasmodium chabaudi* [131] and *Trypanosoma cruzi* [125]. This phenotype is confirmed in IL-10-deficient mice that develop lethal colitis in response to gut flora, as a sign of excessive immune reaction [132]. However, in other situations, the presence of IL-10 was shown to delay or impair protective immune responses. This is supported by studies showing that the absence of IL-10 results in improved clearance of *L. monocytogenes* [133] and *Leishmania major* [134] infections. Therefore, IL-10 is responsible for the fine balance between suppressing and activating immune responses to pathogens. The timing, as well as the level of IL-10 and the immune environment may play a critical role in these processes.

IL-10 in M. tuberculosis Infection

As discussed above, control of *M. tuberculosis* infection requires the activation of specific CD4 T cells in lymph nodes. These cells migrate to the site of infection where they activate macrophages [51], it is therefore expectable a detrimental impact of IL-10 in the course of the disease. Indeed, it has been shown that IL-10 can downregulate the *M. tuberculosis*-induced Th1 responses [135]. Moreover, IL-10 block phagocytosis and impair ROI and RNI production [128]; inhibit IL-12p40 dependent migration of DCs to the lymph nodes [136]; and down-regulate the expression of co-stimulatory molecules by DCs required by T cell differentiation (Figure 3) [137]. These findings suggest that IL-10 can suppress the development of the protective immune response against *M. tuberculosis* [138, 139]. In spite of this, Jung *et al.* described that IL-10 deficient mice had a similar capacity to control *M. tuberculosis* infection when compared to wild-type mice [140]. On the other hand, Higgins *et al.,* showed that IL-10 deficient mice infected with *M. tuberculosis* display exacerbated immunopathology and succumb to disease during late stages of infection [141]. More recently, Redford *et al.,* reported reduced bacterial burden in the lungs and the spleen after M. *tuberculosis* infection in IL-10 deficient mice when compared with wild-type mice [142]. This decrease of bacterial loads is accompanied by an earlier and enhanced Th1 response in lung [142]. While these results are difficult to compare, due to the use of different mycobacterial strains, the dose of infection, the microbial flora of mice from different laboratories, and the time-points assessed they suggest a role for IL-10 in the protective immune response to *M. tuberculosis*.

A different approach to study the role of IL-10 in *M. tuberculosis* is transgenic mice that over-express IL-10. These models show an increased susceptibility to *M. tuberculosis* infection and other mycobacterial species [143]. While transgenic mice that over-express IL-10 in T–cell compartment have enhanced susceptibility to *M. tuberculosis* [143] and BCG [144] infection associated with impaired T cell responses, the increased susceptibility of transgenic mice that over-express IL-10 in the macrophage compartment is associated with macrophage deactivation, but similar T cell responses to that of wild-type mice [145]. These data suggest that, IL-10 may influence the immune response and susceptibility to reactivation disease. In support of these findings, it has been demonstrated that IL-10 is increased in patients with active pulmonary TB [146]. Indeed, T cells co-expressing IFN-γ and IL-10 have been isolated from the bronchoalveolar fluid of TB patients [147]. Moreover, other studies have associated the risk of developing TB with

presence of human polymorphisms related with increased IL-10 responses. One example is *SLC11 A1*, a TB susceptibility locus, that has been associated with increased IL-10 production by monocytes [148]. A recent meta-analysis suggest, an important trend toward polymorphisms in the IL-10 gene and increased susceptibility [149] further supporting an important role for IL-10 during *M. tuberculosis* infection. Future studies will be important to further define its role.

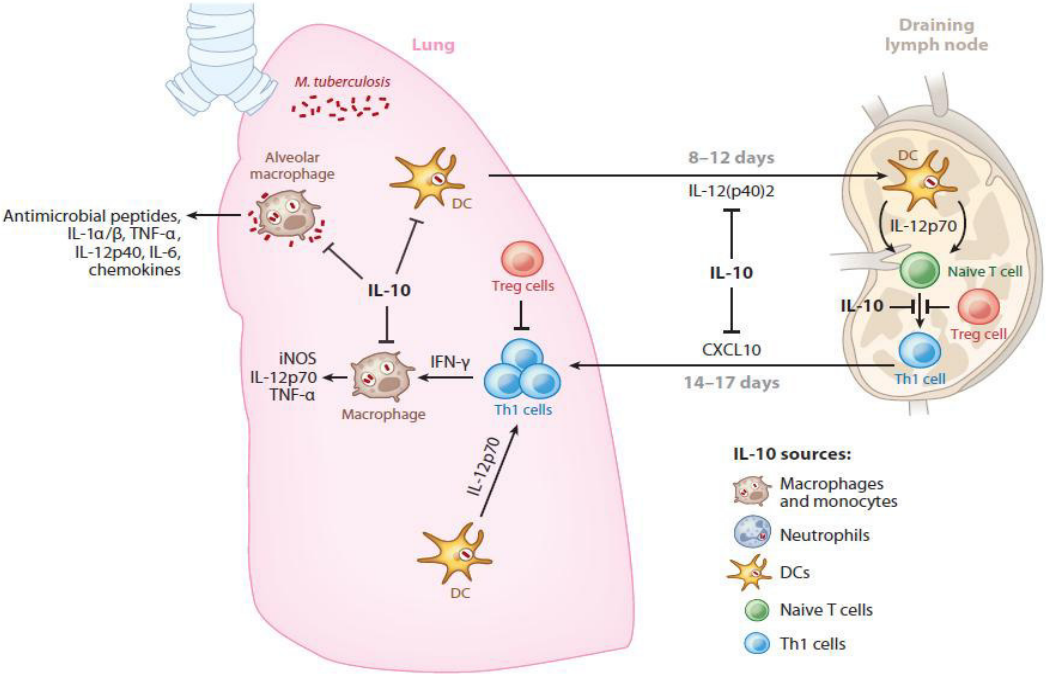

Figure 3. Regulation of the immune response during *M. tuberculosis* infection. Following infection with *M. tuberculosis*, several regulatory actions mediated by IL-10 and Tregs cells serve to limit host-induced immunopathology that may be detrimental to pathogen clearance. The induction of IL-10 during *M. tuberculosis* infection inhibits macrophage and DCs functions, blocking DCs migration to the lymph nodes and subsequently, antigen presentation. In the lymph nodes, IL-10 and Tregs cells can inhibit T cell differentiation and activation of Th1 response. Furthermore, IL-10 can block the migration of these cells to the lung through the downregulation of CXCL10 expression with consequences at macrophage activation levels. From Anne O' Garra *et al.*, The Immune Response in Tuberculosis. Annual Reviews of Immunology,2013

IL-10 in L. monocytogenes infection

As for *M. tuberculosis* infection, the role of IL-10 during *L. monocytogenes* infection is not yet fully defined. Overall, IL-10 appears to have a detrimental role, since IL-10-deficient mice are more resistant to *L. monocytogenes* infection, accompanied by an up-regulation of the Th1 response [133, 150]. However, some studies suggest that IL-10 may have a protective role, possibly associated with reduced immunopathology [151]. Future studies are required to address the role of IL-10 during *L. monocytogenes* infections, specifically in what respects to its impact in the protective T cell response and development of immunopathology.

1.3.2 Sirtuin 2

As discussed above, regulation of the immune response is critical for infection control and host survival. This regulation can be mediated by cytokines but recent data suggest that regulation of gene expression can have a significant impact in the immune response and outcome of the infection [152]. Gene regulation can occur at transcriptional and post-transcriptional levels, translational level and chromatin remodeling. One important modulator of gene expression is the specific modification of histones [153]. Histones are one of the most evolutionary conserved proteins and are essential for the homeostasis of eukaryotic organisms [154]. Each histone can be modified on different residues by phosphorilation, acetylation, metylation and others [154]. The cellular outcome of these modifications depends on the residue target and its cellular environment [154]. Acetylation and deacetylation of nucleosomal histones play an essential role in the modulation of chromatin structure, chromatin function and in the regulation of gene expression [155]. Acetylation is mediated by histone acetyltransferases (HATs), which use acetyl-coenzyme A as a cofactor to catalyze the transfer of an acetyl group to a lysine residue. This modification decreases the affinity between histones and DNA, allowing chromatin to adopt a more relaxed structure (Figure 4). As a consequence, chromatin becomes more permissive to transcription factor binding, activating gene expression [156]. Histones deacetylation, which is mediated by histone deacetylases (HDACs) counteracts the effects of HATs and it is associated with transcriptional repression (Figure 4). These two opposite classes of enzymes need to be tightly controlled to maintain a balanced gene transcription [156]. Indeed, discrepancies in the histone acetylation has been associated with carcinogenesis and cancer progression [155].

Sirtuins are a family of class III HDACs, which use nicotinamide adenine dinucleotide (NAD) as a cofactor (Figure 4) [157]. This family of proteins is highly conserved and is involved in regulation of ribosomal DNA recombination, gene silencing, DNA repair and chromosomal stability and longevity [158, 159]. In mammals, there are seven types of sirtuins described (SIRT1-7), which display diversity in subcellular localization that may account for differences in their biological function and substrate usage [160].

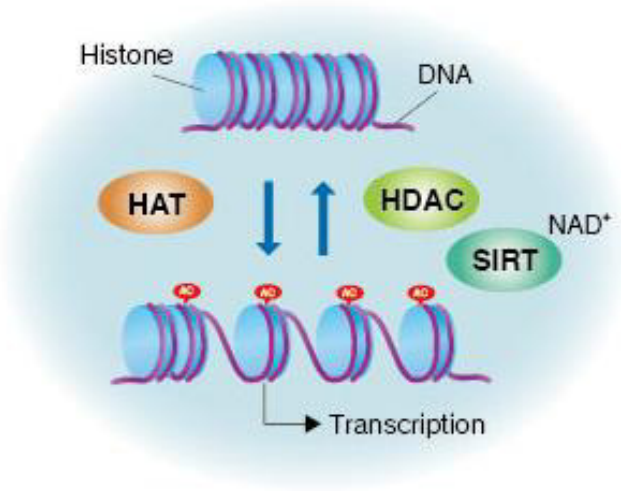

Figure 4 – Gene activation and repression are regulated by acetylation/deacetylation of core histones. Gene expression can be regulated at histones level. Histone acetylation is catalysed by HATs, whereas the reverse reaction is performed by HDACs. When histones are acetylated, interaction of the N terminal of histones with the negatively charged phosphate groups of DNA is decreased. As a consequence, the condensed chromatin is transformed into a more relaxed structure that is associated with increase in gene transcription. In contrast, deacetylation performed by HDACs has the opposite effect. Deactelylation of histone tails become DNA more tightly wrapped around the histone cores, making it harder for transcription factors to bind to the DNA and, consequently, gene transcription. Sirtuin family is HDACs and is involved in gene regulation. From http://ruo.mbl.co.jp/e/product/cyclex/cellular-acetyle.html

Sirtuin type 2 (SIRT2) is the mammalian ortholog of silent information regulator 2 protein (Sir2p) of *Saccharomyces cerevisiae [160]*. SIRT2 is a NAD^+-dependent HDAC that plays an important role in transcriptional silencing [158, 161]. This sirtuin is predominantly a cytoplasmatic protein that may shuttle to the nucleus, for instance during mitosis, where it functions as a mitotic checkpoint protein [162]. The role of SIRT2 has been mainly characterized in the cytoplasm, in regulation of microtubule dynamics once its localize with microtubules [161]. Moreover, SIRT2

has been involved in regulation of cell cycle, DNA repair, apoptosis, metabolism and aging by deacetylation of several transcriptions factors [162]. For example, SIRT2 has involved in regulation of NF-kβ gene expression through deacetylation of p65 [163], regulating the expression of a broad number of target genes involved in the immune and inflammatory response, apoptosis, cell proliferation, differentiation and survival [163].

Several studies have indicated that SIRT2 plays an important role in brain function, inhibiting inflammation and neurotoxicity mediated by microglia [164]. Interestingly, pharmacological or genetic inhibition of SIRT2 has been shown to be protective in Drosophila model of Parkinson's disease [165, 166]. In some types of cancer, as melanoma, gliomas, and gastric carcinomas, levels of SIRT2 are decreased. SIRT2 resides in a genomic region frequently deleted in human gliomas and *in vitro* studies shown that expression of SIRT2 in glioma-derived cell lines markedly reduces their capacity to form colonies [160, 162]. These findings led to the hypothesis that the inactivation of SIRT2 may underlie the development of gliomas and that SIRT2 activation possibly protect against these diseases. Thus, SIRT2 may be a potential target for therapeutic treatments.

There is evidence that histone modifications induced by pathogens can impact host immunity [167]. For instance, i*n vitro* studies have shown that *L. monocytogenes* secret LLO that induce modifications in the host histones [167]. Similar effects were observed with other toxins of the same family, such as perfringolysin of *Clostridium perfringens* and pneumolysin from *Streptococcus pneumonia* [167]. In the mouse, a recent report suggest that *L. monocytogenes* induces SIRT2 translocation from cytosol to the nucleus, causing deacetylation of histone H3 on lysine 18 (H3K18), and impacting the control of infection [168]. These mechanisms of host subversion could be an important strategy used by other pathogens, including *M.* tuberculosis.

AIMS

The immune response against pathogens needs to be tightly regulated to eliminate the pathogen without compromise the host homeostasis. Two important modulators of this balance are IL-10 and SIRT2. The role of IL-10 during *M. tuberculosis* infection or *L. monocytogenes* infection remains controversial, and it is still not well understood whether this cytokine plays a role in host resistance or susceptibility. As for SIRT2, it has been recently suggested that the activation of this histone deacetylase is an active mechanism that *L. monocytogenes* exploits to establish infection. We are interested in determining whether or not *M. tuberculosis* also uses this pathway to promote infection.

To address the role of IL-10, we used a novel model of IL-10-over-expression, the pMT-10 mouse model that allows us to evaluate the impact of a transiently IL-10 over-expression during different phases of the immune response. To address the role of SIRT2 we used mice that are deficient in the gene encoding this enzyme in the myeloid lineage.

The main goals of this thesis are:

(i) Determine the impact of transient high levels of IL-10 in the immune response and the outcome of aerosol *M. tuberculosis* infection; specifically, we will focus on the impact of IL-10 at the time of infection, during the acute phase and during the chronic phase of *M. tuberculosis* infection.

(ii) Determine the role of IL-10 in the mechanisms of immune-mediated protection and outcome of *L. monocytogenes* infection.

(iii) Define the role of SIRT2 in the *in vitro* response of macrophages and the *in vivo* outcome of the *M. tuberculosis* infection.

MATERIAL AND METHODS

Animals. pMT-10 mice on a C57BL/6 background were produced by Drs. António G. Castro and Paulo Vieira at the Gulbenkian Institute of Science, Oeiras (IGC). A mouse IL-10 cDNA sequence was cloned in the p169ZT vector containing the sheep metallothioneinc (MT) Ia promoter, a β-globin splice site and the SV40 polyadenylation signal. The resulting vector (pMT-IL10) was injected in C57BL/6 eggs and the transgenic were identified by PCR using MT and IL-10 specific primers. The association of an MT promoter with the mouse IL-10 cDNA sequence allows for the over-expression of IL-10 in the presence of zinc. IL-10 over-expression was induced by administration of 2% sucrose solution containing 50 mM of zinc sulfate in the animals' drinking water, *ad libitum.* A group of transgenic littermates were supplied with 2% sucrose solution as a control.

LysM-Cre+Sirt2 flox+/flox+ mice were obtained by Teresa F. Pais at IMM by crossing LysMcre mice (The Jackson Laboratory) with Sirt2 floxed mice, which were used through an MTA with Johan Auwerx & Kristina Schoonjans Laboratory of Integrative and Systems Physiology, NCEM, Ecole Polytechnique de Lausanne (EPFL), Switzerland. Experimental mice were matched for sex and age and were infected at between 8 to 12 weeks of age. All procedures involving live animals were carried out in accordance with the European Union Directive 86/609/EEC, and previously approved by the Portuguese National authority *Direcção Geral de Veterinária*.

Bacteria and Infection. *M. tuberculosis* H37Rv, originally from the Trudeau Institute Mycobacterial Collection, was grown in Middlebrook 7H9 liquid media (BD Biosciences) for 7–10 days and then diluted into Proskauer Beck (PB) medium supplemented with 0.05% Tween 80 and 2% glicerol to the mid-log phase. Bacterial stocks were aliquoted and frozen at –80°C. To determine the concentration of *M. tuberculosis* aliquots, 10-fold serial dilutions of 6 frozen vials were plated in Middlebrook 7H11 (BD Biosciences) agar plates supplemented with 10% oleic acid/albumin/dextrose/catalase (OADC) and 0.5% glycerol and viable bacteria were determined after 3 weeks post-incubation at 37°C.

Mice were infected with *M. tuberculosis* H37Rv via the aerosol route using an inhalation exposure system (Glas-Col). Briefly, the mycobacterial inoculum was prepared to a concentration of $2x10^6$ CFUs from the frozen original stock, by passing it through a 26G needle 4-6 times to disrupt bacterial clumps and diluted in water (Aqua B. Braun). Mice were exposed to the aerosol over a period of 40 minutes with 100 to 200 *M. tuberculosis* bacteria delivered to the lungs. The

infection dose was confirmed by counting of viable bacteria in the entire lung of 5 animals 3 days after the aerosol infection.

L. monocytogenes strain EGD was grown in brain heart infusion broth (BHIB) for 24h, washed twice with PBS, aliquoted and frozen in PBS containing 30% glycerol at -80°C. To determine the concentration of *L. monocytogenes* aliquots serial dilutions of 1 frozen vial were plated in BHI medium plates and the viable bacteria were determined after 24 hours post-incubation at 37°C. The 50% lethal dose (LD50) of this inoculum was approximately $1x10^6$ when administered through the intraperitoneal (i.p.) route to wild-type C57BL/6 mice. For experimental infections, mice were intraperitoneally infected with $5x10^5$ bacteria diluted in 200µl of phosphate-buffered saline (PBS).

***In vivo* IFN-γ neutralization.** *In vivo* IFN-γ blockade was carried out by i.p. injections of 0.5 mg of anti-Mouse IFN-γ (clone XMG1.2, eBiosciense) twice a week, as described in the respective figure legends.

Preparation of Single Cell Suspensions. At selected time-points post-infection, mice were killed by CO_2 asphyxiation and the organs were aseptically excised. Lungs were first perfused with PBS through the right ventricle of the heart to exclude blood cells, dissected with 2 sterile scalpels and incubated at 37°C with collagenase IX (0.7mg/ml, from Sigma-Aldrich) for 30 minutes. Single cell suspensions from the digested lungs, liver, spleen or mediastinal lymph nodes were homogenized and passing through a 40-µm-pore-size nylon cell strainer (BD Biosciences). Lymph nodes cells were resuspended in cDMEM and used for bacterial load determination, flow cytometry analysis and RNA extraction. Liver cells were resuspended in cDMEM and used for bacterial burden determination. Lung cells were treated with erythrocyte lyses solution (0.87% of NH_4Cl solution and 5% of PBS in water) to lyse residual red blood cells and resuspended in cDMEM and used for bacterial burden determination, flow cytometry analysis or RNA extraction. Single cell suspensions were counted using a Countess® Automated Cell Counter (Life Technologies).

Measurements of Survival. Mice infected with *L. monocytogenes* were monitored twice daily and when the animals had a behavior unresponsive or recumbent were considered moribund and euthanized.

Bacterial Load Determination. To determine the *M. tuberculosis* bacterial burdens, lung, liver or mediastinal lymph nodes single cell suspensions were incubated with 0.1% saponin (Sigma) for 10 min to release intracellular bacteria. The number of CFU was determined by plating 10-fold serial dilutions of the disrupted cell suspensions in Middlebrook 7H11 agar plates supplemented as described above. BBL™ MGIT™ PANTA™ antibiotic mixture (BD Bioscience) was used to prevent contaminations. Viable mycobacteria colonies were counted after 3 weeks of incubation at 37°C. For control of *L. monocytogenes* bacterial burden, spleen and liver were collected and placed in cold PBS. The liver and the remaining spleen were disrupted by high pressure using a homogenizer releasing the bacteria to the solution. The number of *L. monocytogenes* CFU was measured by plating of 10-fold serial dilutions of homogenate tissue onto BHI agar plates and incubating over-night (ON) at 37°C.

Flow Cytometry Analysis. For intracellular cytokine staining, 2-3x10^6 cells were stimulated with a mixture of phorbol myristate acetate (PMA) (50ng/ml) and ionomycin calcium salt (4µg/ml), in the presence of brefeldin A (10µg/ml) (all from Sigma-Aldrich) for 4 hours at 37°C. After stimulation, the cells were recovered and fixed ON with 10% neutral buffered formalin. Cells were pre-treated with Fc block (anti CD16/CD32) for 10 minutes to minimize a non-specific antibody binding and permeabilized with saponin FACS buffer (PBS containing 2% of FBS, 0.01% of azide and 0.5% saponin) for 10 minutes and stained for surface and intracellular antigens for 30 minutes at room temperature (RT). The following antibodies were used: CD3-PerCPcy5.5 (clone 145-2C11, eBioscience), CD4-APC-Cy7 (clone GK1.5, eBioscience), CD8-FITC (clone 5H10-1, Biolegend), CD11b-PE (clone M1/70, eBioscience); CD11c-BV421 (clone N418, Biolegend); CD19-APC (clone eBio1D3, eBioscience), Ly6G-APC (clone 1A8, Biolegend); MHC II-FITC (M5/114.15.2, Biolegend); Ly6C-PerCPCy5.5 (clone AL-21, Pharmingen); IL-17-APC (clone TC11-18H10.1, eBioscience), IFN-γ-PECy7 (clone XMG1.2 from Biolegend), iNOS-FITC (6/iNOS/NOS Type II, eBioscience). Samples were acquired on a LSRII flow cytometry with Diva Software. All data were analysed using FlowJo version 7 software. The total number of cells in each gate was calculated using the total number of cells determined by Countess® Automated Cell Counter.

Differentiation and Infection of Bone Marrow-Derived Macrophages (BMDM). Bone marrow cells were flushed from tibiae and femurs' mice with complete Modified Eagle Medium (cDMEM, DMEM supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 1% of HEPES 1M, 1% L-glutamine and 1% sodium pyruvate 100mM (all from GIBCO)). Bone marrow cells were counted and cultured in cDMEM supplemented with 20% L929-cell-conditioned medium (LCCM) in 8-cm plastic petri dishes (Sterilin) in 8ml at a concentration of 0.5 x 10^6 cells/ml and cultured during 7 days under 37°C and 5% CO_2 conditions. On day 4 of differentiation, 10ml of cDMEM supplemented with 20% LCCM were added to the cultures. At day 7, adherent cells were scraped, harvested and counted using Neubauer chamber. After, BMDM were plated at a concentration of 1x10^6/200µl in 24-wells plates (Nunc) and incubated at 37°C. The supernatant was recovered and the cells were infected with *M. tuberculosis* at a multiplicity of infection (MOI) of 2:1 (bacteria/macrophage ratio) and incubated at 37°C. Four hours post-infection, cells were washed 3 times with incomplete DMEM to remove the bacteria that were not internalized. Washed cells were ressuspended in 0.5 ml of cDMEM and incubated at 37°C for 72 hours or used to determine bacterial internalization. For that, 0.1% saponin (Sigma) in PBS was added to the wells and the cells were incubated at RT for 10 minutes to release the intracellular bacteria. The number of viable bacteria was determined by plating 10-fold serial dilutions of the disrupted cell suspensions in supplemented Middlebrook 7H11, as described before. Viable mycobacteria colonies were determined after 3 weeks of incubation at 37°C.

For evaluate cytokine production, cells were plated at a concentration of 0.5x10^6/well in 24-wells plates and infected with *M. tuberculosis* at MOI of 2:1 in a final volume of 0.5ml and incubated at 37°C. Twenty-four hours post-infection, supernatants were recovered, filtered with 0.2 µm filter and stored at -80°C.

Cytokine determination by ELISA. The concentration of IL-10 in the serum of mice and IL-6 and TNF-α in the supernatants was determined by using the commercially available ELISA kit for IL-10 (88-7104), IL-6 (88-7064) and TNF-α (88-7324), all from eBioscience, according to the manufacturer's instructions.

RNA extraction and quantification. Total RNA from cell suspensions was extracted by using TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Briefly, glycogen (20µg/µl from Roche) was added to each sample and incubated for 5 minutes

at RT. After incubation, 50µl of chloroform (Sigma-Aldrich) were added and the samples were mixed by vortexing and incubated on ice for 15 minutes. After, samples were centrifuged at 13000 rpm for 15 minutes at 4°C, and the upper aqueous phase, containing the RNA, was carefully recovered to a new tube, and mixed with an equal volume of isopropyl alcohol (Sigma-Aldrich) to precipitate the RNA. Samples were incubated ON at -20°C and centrifuged at 13000 rpm for 15 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 800µl of 70% ethanol (Carlo Erba reagents). Ethanol was completely removed after centrifugation at 9000 rpm for 5 minutes and the dried RNA pellet was resuspended in RNase/DNase-free water (Gibco). RNA concentration was measured at 260nm (Nanodrop ND-1000 Spectrophotometer) and the purity assessed through the A_{260}/A_{280} and A_{260}/A_{230} ratios.

Complementary DNA synthesis. Complementary DNA (cDNA) was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, reaction mix was composed by 4µl of Reaction buffer, 1µl of OligodT, 2µl of dNTP, 1µl of diethylpyrocarbonate (DEPC) water, 1µl of Riboblock and 1µl of Reverter Aid, per sample and mixed with 10µl of RNA sample. The cDNA synthesis reaction was performed in thermocycler (MyCycler, Thermal Cycler from Bio-Rad) with the following program: 42 °C for 60 minutes following by 15 minutes at 70°C. The resultant cDNA template was used for quantification of target genes expression by real-time PCR (RT-PCR) analysis using SYBR green or TaqMan detection systems.

REAL-TIME PCR. For SYBR Green reactions 1µl of each cDNA sample was mixed with 9µl of reaction mix that contains 3µl of water, 1µl of 0.4µM forward and reverse specific primer and 5µl of SYBR green qPCR Master Mix (Fermentas). RT-PCR was performed in CF*96TM Real-time system (Bio-Rad) using the following program: 95°C for 15 minutes, followed by 40 amplification cycles of 95°C for 15 seconds, 58°C for 20 seconds and 70°C for 15 seconds, and the melting curve analysis. For TaqMan reactions 1µl of each cDNA sample was mixed with 9µl of reaction mix that contains 3.5µl of water, 0.5µl of specific primer-probes and 5µl of TaqMan Gene Expression Master Mix (Applied Systems). The RT-PCR program used was 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative mRNA expression of the target gene was normalized to the levels of the housekeeping gene using the ΔCt method: 1.8^(Housekeeping gene mRNA expression – Target gene mRNA expression) x

100000. The sequences of primers and the references of the TaqMan primer-probe sets used in RT-PCR are listed in Table 1.

Table I: Sequences of SYBR Green primers and TaqMan primer-probes used.

SYBR Green primers	Sense	Antisense
Ubiquitin	5'-TGGCTATTAATTATTCGGTCTGCAT-3'	5'-GCAAGTGGCTAGAGTGCAGAGTAA-3'
p19	5'- CGTATCCAGTGTGAAGATGGTTGT -3'	5'- GCTCCCCTTTGAAGATGTCAGA -3'
IL-6	5'-ACACATGTTCTCTGGGAAATCGT6-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'

TaqMan Primer-Probes	
HPRT	Mm.299281
MIP-2	Mm.436450
CXCL9	Mm.434946
CXCL10	Mm.445235
CXCL11	Mm.444662

Histology. The right upper lobe of the lung and the right and upper part of liver was inflated and maintained for 1 week in 3.7% phosphate-buffered formalin. After, these organs were embedded in paraffin, sectioned in 3μm thickness sections, and stained with hematoxylin and eosin (HE).

Statistical Analysis. The results are given as means ± standard error of the mean (SEM) of at least four animals per experimental group, as indicated in the figure legends. Differences between groups were analyzed by Student T Test or Two away ANOVA and the post test of Bonferroni using Graph Pad Prism 5 software, as indicated in the figure legends. Values were considered significant for $p \leq 0.05$ and represented with * for $p \leq 0.05$; ** for $p \leq 0.01$ and *** for $p \leq 0.001$.

RESULTS – PART I

Impact of IL-10 over-expression in chronic and acute infections

by intracellular bacteria

1.1 Effect of IL-10 over-expression during the early stages of the immune response against *M. tuberculosis* infection

Early immunity against *M. tuberculosis* infection includes several critical events for the control of infection, including macrophage and DCs activation, APCs trafficking from lung to the lymph nodes and T cells activation [51]. Several reports have shown that IL-10 regulate these events to limit immunopathology, without compromising pathogen clearance [125]. Indeed, IL-10 has been described to downregulate direct and indirectly macrophages and DCs function, and T cell priming [51, 125, 169]. In this regard, we asked whether IL-10 over-expression before *M. tuberculosis* challenge and during the early innate phase of the immune response would impact the outcome of the infection. To test this hypothesis, a group of pMT-10 mice was induced to over-express IL-10 between day 3 before *M. tuberculosis* challenge and day 5 post-infection, a period in which the mycobacteria is recognized by alveolar macrophages in the lung and innate immune response is initiated. The IL-10 promoter was induced by administration of zinc sulfate solution, in the drinking water, and two groups were analysed – infected and induced pMT-10 mice (pMT-10 + Zn) and, as a control, infected and non-induced pMT-10 mice (pMT-10). As previously reported, IL-10 levels could be detected 3 days after zinc administration and two days after the end of the zinc administration and in control pMT-10 mice, circulating IL-10 was not detect (data not shown).

IL-10 over-expression during day 3 before M. tuberculosis infection and day 5 post-infection does not impact the outcome of infection

At different time-points after *M. tuberculosis* infection, lungs and lymph nodes were recovered and bacterial loads were compared between control pMT-10 and induced pMT-10 mice. After aerosol infection, induced pMT-10 mice showed similar bacterial burden when compared to control pMT-10 mice, over the course of infection (Figure 1a). In the lymph nodes, the same trend was observed (Figure 1b). High IL-10 levels were detected in the serum of induced pMT-10 mice whereas in control pMT-10 mice IL-10 was bellow the detection level (Figure 1c). These data suggest that an early IL-10 over-expression does not impair the host resistance to *M. tuberculosis* infection.

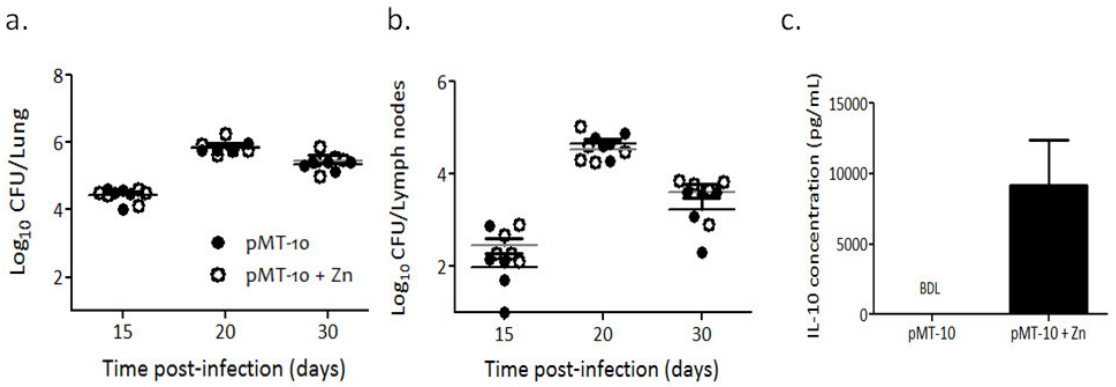

Figure 1. Early IL-10 over-expression does not impact the control of aerosol *M. tuberculosis* infection. pMT-10 mice exposed to zinc between day 3 before *M. tuberculosis* infection and day 5 post-infection (open circles) and pMT-10 control littermates (closed circles) were infected with 100 to 200 CFU of *M. tuberculosis* H37Rv via the aerosol route. The bacterial burden on day 3 post-infection was Log_{10}(CFU)=2.05±0.08 (Mean ± SEM for five lungs). At the indicated time points, lung **(a.)** and lymph nodes **(b.)** cell suspensions were prepared, diluted and plated into Middlebrook 7H11 medium to determine the number of viable mycobacteria. **(c.)** At day 3 post-infection, blood was harvested from infected animals and IL-10 concentration in the serum of the induced pMT-10-mice and pMT-10 control group was determined by ELISA. Data points show the Mean ± SEM for five mice per group and bars show the Mean ± SEM from one experiment of 5 mice per group. The statistical analysis between the different groups was determined by the Two-way ANOVA and the post test of Bonferroni.

IL-10 over-expression between day 3 before M. tuberculosis infection and day 5 post-infection causes a delay in the accumulation of CD4 T cells in the lungs of M. tuberculosis infected mice

Despite the observation that high levels of IL-10 in the very early phase of the immune response did not impact the control of infection, we next investigated whether these high IL-10 levels impacted the course of the T cell response. To assess this, at the indicated time-points after *M. tuberculosis* infection, lungs and lymph nodes were removed and stimulated with PMA and ionomycin, in the presence of brefeldin A, to induce cellular activation and cytokine production. The lung and lymph node cells were stained with antibodies to measure Th1 and Th17 cell responses. Viable cells were gated and CD4 T cells selected based on IFN-γ (Th1) or IL-17 (Th17) production. The zebra plots show the electronic gate used to identify CD4 T cells gated on single cells (Figure 2a) and IFN-γ-producing CD4 T cells (Figure 2b) and IL-17-producing CD4 T cells (Figure 2c). The total number of CD4 T cells in the lungs of both induced pMT-10 and control pMT-10 mice followed similar kinetics over time (Figure 2d). However, at day 20 post-infection,

there was reduced numbers of CD4 T cells in the lungs of induced pMT-10 mice (Figure 2d). Moreover, IFN-γ-producing T cells followed an identical kinetics over time (Figure 2e), whereas IL-17-producing CD4 T cells were decreased at day 20 post-infection, in the presence of high levels of IL-10 (Figure 2f).

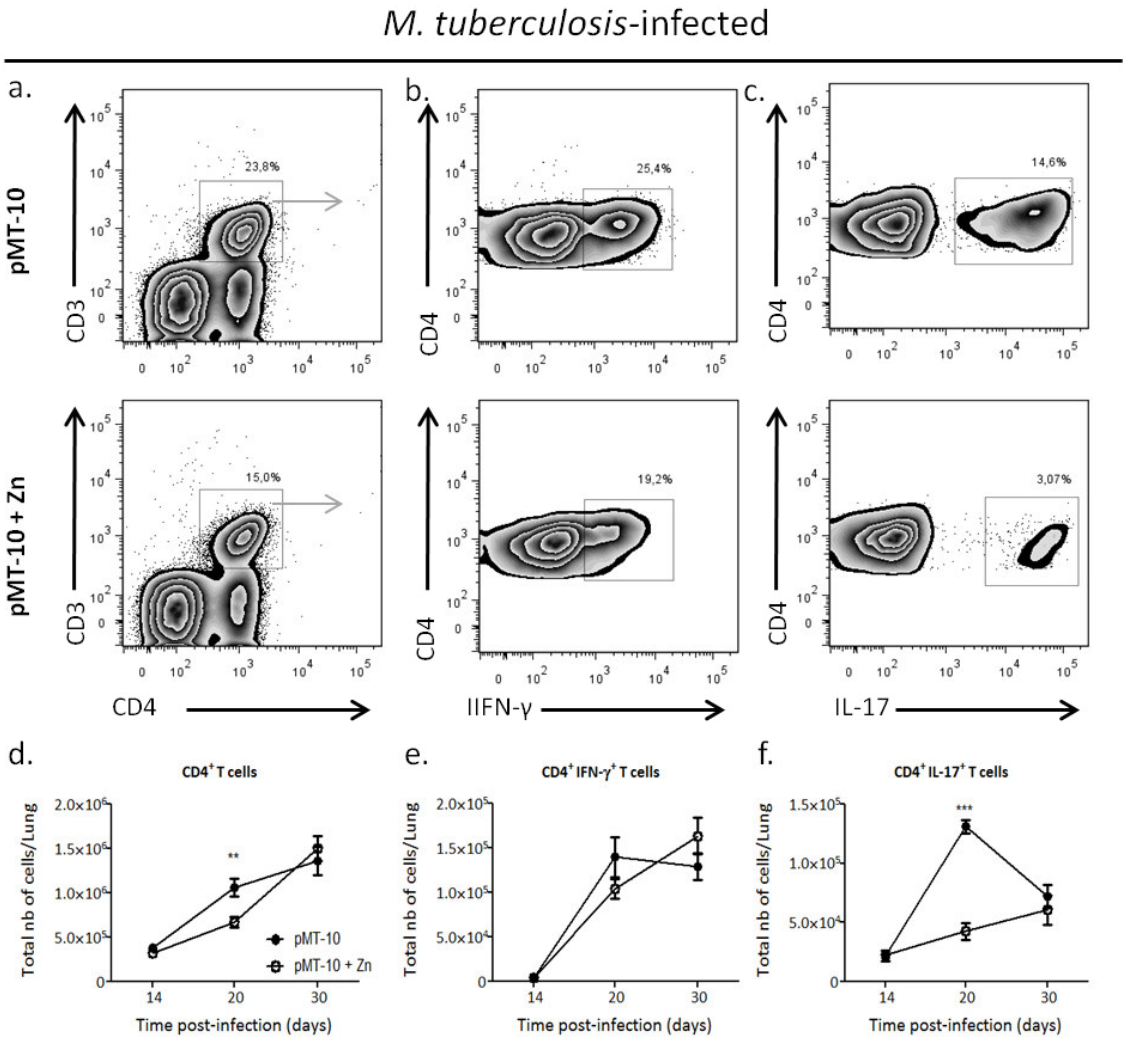

Figure 2. Early IL-10 over-expression induces a delay in the accumulation of T cells in the lung during *M. tuberculosis* infection. pMT-10 mice exposed to zinc between day 3 before *M. tuberculosis* infection and day 5 post-infection (open circles) and pMT-10 control littermates (closed circles) were aerosol infected with *M. tuberculosis* H37Rv as in Figure 1. At the indicated time points post-infection, lung cell suspensions were stimulated with PMA and ionomycin for 4 hours, in the presence of brefeldin A. After this, cells were overnight fixated, stained with specific antibodies for CD3, CD4, IL-17 and IFN-γ and analyzed by flow cytometry. The zebra plots show the electronic gate used to identify CD4 T cells population gated on single cells **(a.)** and IFN-γ- **(b.)** and IL-17- **(c.)** producing cells within CD4 T cells. Numbers indicate percentage of CD4 T cell populations from this experiment. The total number of CD4+ T cells **(d.)** making IL-17 **(e.)** and IFN-γ **(f.)** was determined. Data points show the Mean ±

SEM from one experiment of 5 mice per group. The statistical analysis between the different groups was determined by the Two-way ANOVA and the post test of Bonferroni (**p≤0.01; ***p≤0.001).

This slight and transient delay in CD4 T cell accumulation in the presence of high levels of IL-10 may be related with a delay in the activation of CD4 T cells in the lymph nodes since that the total number of CD4 T cells in the lymph nodes was decreased when IL-10 was over-expressed (data not shown).

1.2 Effect of IL-10 over-expression during the chronic stage of the immune response against *M. tuberculosis* infection

The host innate immune response to *M. tuberculosis* infection is essential for the initial defense against bacillus, but the adaptive immunity is required for containment of the infection during the chronic stage [51]. To address whether IL-10 over-expression during the chronic phase of the immune response against *M. tuberculosis* impacts granuloma stability and TB reactivation, we infected pMT-10 mice with *M. tuberculosis* via the aerosol route and induced IL-10 over-expression between day 50 and day 57 post-infection.

IL-10 over-expression during day 50 and day 57 after M. tuberculosis infection does not impact the outcome of infection

At different time-points after IL-10 induction, lung and liver were collected and the bacterial loads determined. As represented in figure 3a, lungs from induced pMT-10 mice had similar bacterial burdens as control pMT-10 mice, over the course of infection. However, the livers of induced pMT-10 mice at day 57 post-infection had a small but significant increase in bacterial loads when compared to that of control pMT-10 mice. As the infection progressed, a similar bacterial burden was observed (Figure 3b). To confirm that IL-10 was over-expressed in induced pMT-10 mice, IL-10 amounts were measured in the serum of mice. As expected, high IL-10 levels could be detected at day 57 post-infection in induced pMT-10 mice but not in control mice (Figure 3c). These data suggest that, a later IL-10 over-expression does not significantly impair the control of *M. tuberculosis* infection.

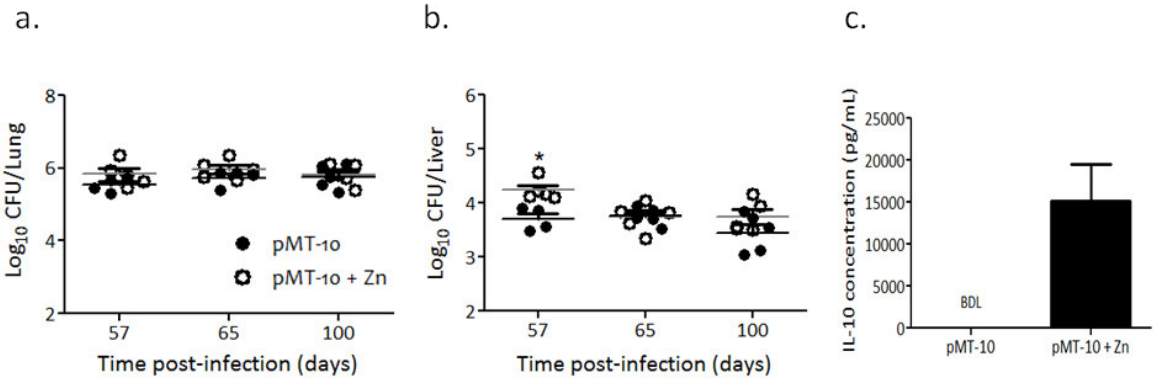

Figure 3. Late IL-10 over-expression does not impact the control of aerosol *M. tuberculosis* infection. pMT-10 mice exposed to zinc between day 50 and day 57 post-infection (open circles) and pMT-10 control littermates (closed circles) were aerosol infected with *M. tuberculosis* H37Rv as in Figure 1. The bacterial burden on day 3 post-infection was Log10(CFU)=2.17±0.06 (Mean ± SEM for five lungs). At the indicated time points, lung **(a.)** and liver **(b.)** cell suspensions were prepared, diluted and plated into Middlebrook 7H11 medium to determine the number of viable mycobacteria. **(c.)** At day 57 post-infection, blood was harvested from infected animals and IL-10 concentration in the serum of the induced pMT-10 mice and pMT-10 control group was determined by ELISA. Data points show the Mean ± SEM for five mice per group and bars show the Mean ± SEM from one representative of three experiments of 5 mice per group. The statistical analysis between the different groups was determined by the Two-way ANOVA and the post test of Bonferroni (*p<0.05).

IL-10 over-expression between day 50 and day 57 after M. tuberculosis infection does not impact the kinetics of lymphoid cells in the lungs of M. tuberculosis infected mice

We next asked whether the high levels of IL-10 during chronic phase impacted the T and B cell responses. The CD8 T cell response has been described as important in the control of bacteria during the chronic phase of *M. tuberculosis* infection [170] and CD4 T cells have been demonstrated to be involved in protection against *M. tuberculosis*, as shown by the increased rates of TB reactivation in HIV-infected individuals [44]. Furthermore, B cells have also been implicated for the organization and development of granulomatous lesions in the lung of mice infected with *M. tuberculosis* [171]. To address the impact of IL-10 in these cells, lungs were removed and stained with extracellular antibodies to T and B cells. Viable cells were gated, following CD19 B cells or CD3 T cells and, within these, CD4 or CD8 T cells were analyzed. The total number of T and B cells in the lungs of induced and control pMT-10 mice were similar over

time (Figure 4) suggesting that the presence of high levels of IL-10 during the chronic stage of *M. tuberculosis* infection does not significantly impact the protective response.

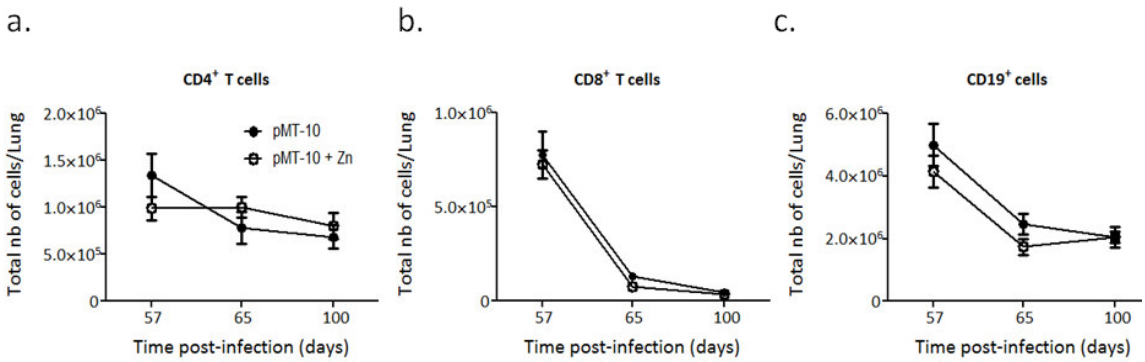

Figure 4 - Late IL-10 over-expression does not impact the kinetics of T and B cells in the lung during *M. tuberculosis* infection. pMT-10 mice exposed to zinc between day 50 and 57 post-infection (open circles) and pMT-10 control littermates (closed circles) were aerosol infected with *M. tuberculosis* H37Rv as in Figure 1. At the indicated time points post-infection, lungs were prepared and stained with specific antibodies for CD3, CD4, CD8 and CD19 and analyzed by flow cytometry. The total number of CD3+ CD4+ T cells **(a.)**, CD3+ CD8+ T cells **(b.)** and CD19+ cells **(c.)** were determined. Data points show the Mean ± SEM from one experiment of 5-6 mice per group. The statistical analysis between the different groups was determined by the Two-way ANOVA and the post test of Bonferroni.

1.3 Effect of IL-10 over-expression prior to T cell response to *M. tuberculosis* infection

The antigen presentation is an important step in the host protective response to *M. tuberculosis* infection. Indeed, APCs present antigens through MHC class II to induce CD4 T cell responses which, as discussed above, are crucial for the control of *M. tuberculosis* infection [172]. It is well established that IL-10 can downregulate MHC class II expression, thereby compromising the protective immune response [129]. We investigated whether IL-10 over-expression between days 7 and 14 of infection, corresponding to the phase when T cells are primed, impacts the outcome of *M. tuberculosis* infection. To address this, pMT-10 mice infected with *M. tuberculosis* via the aerosol route were induced to over-express IL-10 between day 7 and day 14 post-infection.

L-10 over-expression during day 7 and day 14 does not compromise the control of M. tuberculosis infection

At different time-points after aerosol *M. tuberculosis* infection, lung and lymph nodes of *M. tuberculosis*-infected mice, were collected for bacterial burden determination. We observed that, bacterial burdens in the lungs of induced pMT-10 mice were similar to that of control pMT-10 mice (Figure 5a). Similarly, in the lymph nodes we observed that induced pMT-10 mice had equal bacterial burden as control pMT-10 mice (Figure 5b). To confirm that IL-10 was indeed over-expressed in induced pMT-10 mice, IL-10 levels were measured in the serum. As expected, IL-10 levels could be detected at day 14 post-infection in induced pMT-10 mice but not in control mice (Figure 5c). Our data suggest that IL-10 over-expression prior T cell activation does not impair the control of *M. tuberculosis* infection.

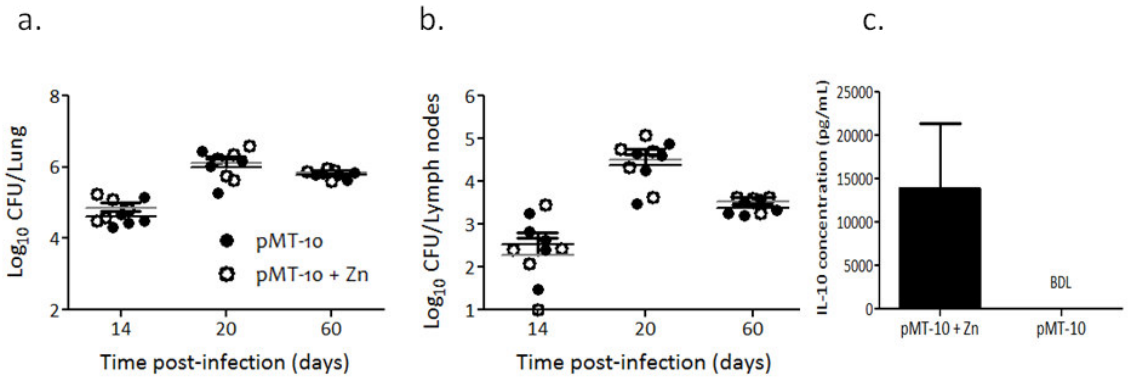

Figure 5. IL-10 over-expression between day 7 and day 14 does not impact the control of aerosol *M. tuberculosis* infection. pMT-10 mice exposed to zinc between day 7 and day 14 post-infection (open circles) and pMT-10 control littermates (closed circles) were aerosol infected with *M. tuberculosis* H37Rv as in Figure 1. The bacterial burden on day 3 post-infection was Log10(CFU)=2.20±0.10 (Mean ± SEM for five lungs). At the indicated time points, lung **(a.)** and lymph nodes **(b.)** cell suspensions were prepared, diluted and plated into Middlebrook 7H11 medium to determine the number of viable mycobacteria. **(c.)** At day 14 post-infection, blood was harvested from infected animals and IL-10 concentration in the serum of the induced pMT-10 mice and pMT-10 control group was determined by ELISA. Data points show the Mean ± SEM for five mice per group and bars show the Mean ± SEM from one representative of three experiments of 5 mice per group. The statistical analysis between the different groups was determined by the Two-way ANOVA and the post test of Bonferroni.

IL-10 over-expression between day 7 and day 14 after M. tuberculosis infection differently impacts the CD4 T cell response in the lungs

We next investigated whether IL-10 over-expression during T cell priming impacted the CD4 T cell response to *M. tuberculosis* infection. To address this, lungs and lymph node cells from *M. tuberculosis*-infected and uninfected mice were stimulated with PMA and ionomycin, in the presence of brefeldin A. Cells were stained with specific antibodies for CD3, CD4, IFN-γ and IL-17. Viable cells were gated and selected for CD4 T cells that were further characterized by IFN-γ (Th1) or IL-17 (Th17) production. The zebra plots show the electronic gate used to identify IFN-γ-producing CD4 T cells and IL-17-producing CD4 T cells on gated CD4 T cells (Figure 6a and b). Both *M. tuberculosis*-infected and uninfected mice were induced to over-express IL-10 for 7 days (between day 7 and day 14 post-infection for infected mice). At the end of zinc administration, we observed that in the uninfected induced mice the total number of IL-17-produncing CD4 T cells in the lungs was slightly lower than in pMT-10 control mice, but did not reach statistical significance (Figure 6c). In *M. tuberculosis*-infected mice we observed the same trend, but now reaching statistical significance (Figure 6d). Interestingly, for the IFN-γ-response, we observed that in both uninfected and *M. tuberculosis*-infected induced pMT-10 mice the total number of IFN-γ-producing CD4 T cells was higher in the presence of high levels of IL-10 (Figure 6e and f). This different number of CD4 T cells in the presence of high levels of IL-10 is likely not related with an impact of IL-10 in the activation of CD4 T cells in the lymph nodes, since the total number of CD4 T cells in the lymph nodes was not altered in the presence of high levels of IL-10 (data not shown). These data suggest an important and intriguing role for IL-10 in the lung CD4 T cell response. Since both infected and uninfected mice had a similar response, we conclude that the altered T cell response was not completely mediated by the *M. tuberculosis* infection.

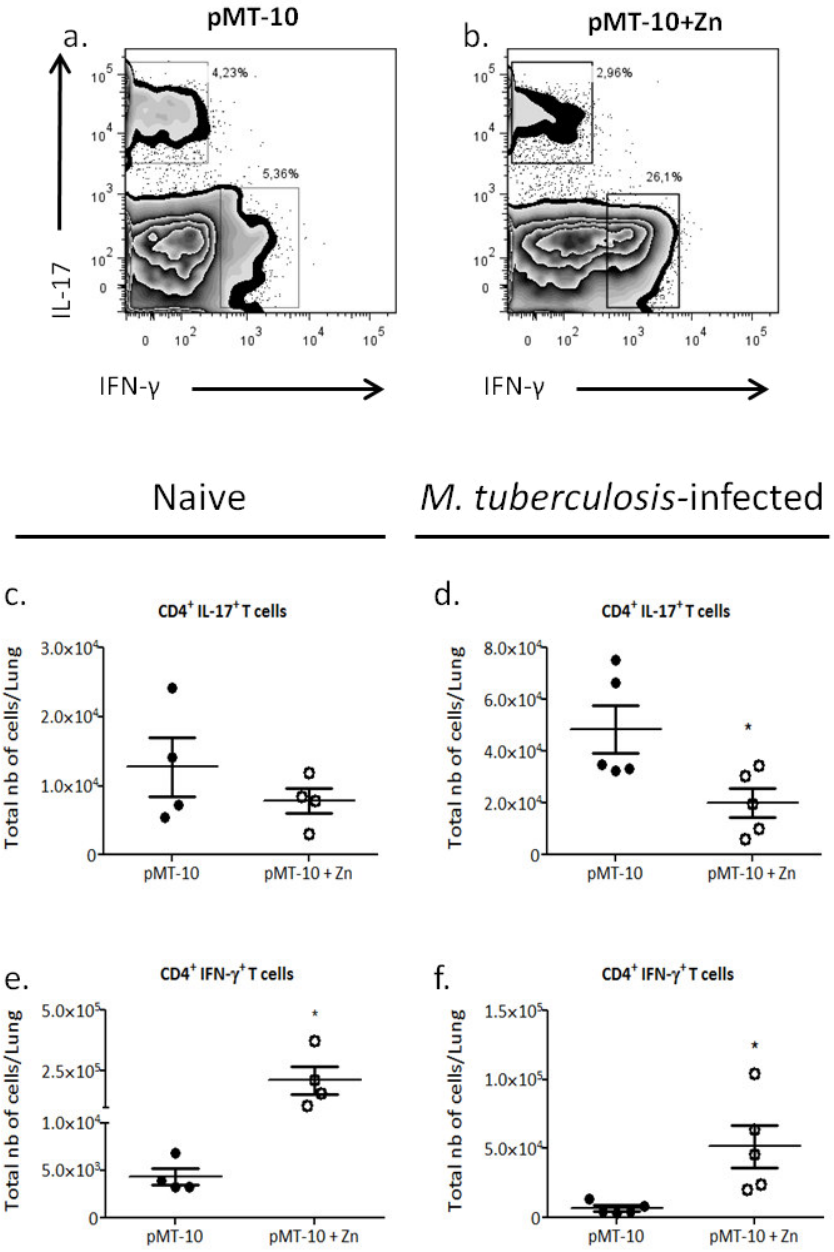

Figure 6. IL-10 over-expression for seven days differently impacts the CD4 T cell response. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (open circles) and pMT-10 control littermates (closed circles) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, lung cell suspensions from uninfected (left panels) and *M. tuberculosis*-infected mice (right panels) were prepared and restimulated for 4h with PMA, ionomycin and brefeldin A. After restimulation, cells were stained with specific antibodies for CD3, CD4, IL-17 and IFN-γ and analyzed by flow cytometry. **(a. and b.)** The zebra plot show the electronic gate used to identify IFN-γ-producing or IL-17-producing T cells gated on CD3+ CD4+ cells. Numbers indicate percentage of CD4+ IFN-γ+ and CD4+ IL-17+ cells. **(c. to f.)** Total numbers of CD4+ IL-17+ **(c. and d.)** and CD4+ IFN-γ+ **(e. and .f)** were determined. Data points show the Mean ± SEM from one representative experiment of 3 experiments with 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (*p<0.05).

To address whether the impact of IL-10 in the IL-17 response was directly in the T cells or mediated by reducing the expression of cytokines important in the development of this phenotype, we measured the expression of *Il23a* and *Il6*. At the end of zinc administration, the expression of *Il23a* appeared to be slightly lower in the induced pMT-10 mice, but did not reach statistical significance (Figure 7a and b). Regarding the *Il6* expression, both infected and uninfected mice had similar levels of *Il6* expression independently of IL-10 levels (Figure 7c and d). We also looked at the expression of *Cxcl2,* a chemokine expressed mostly by Th17 cells, important for the recruitment of neutrophils. As shown above for the IL-17 response we found that, *Cxcl2* expression was similar in both groups of uninfected mice but significantly reduced during *M. tuberculosis* infection in induced pMT-10 mice when compared to control mice (Figure 7e and f). Altogether, our data suggest that the decrease of total number of IL-17-producing CD4 T cells in the presence of high levels of IL-10 is not related with reduced *Il23a* expression but may be a direct effect of IL-10 in the T cells.

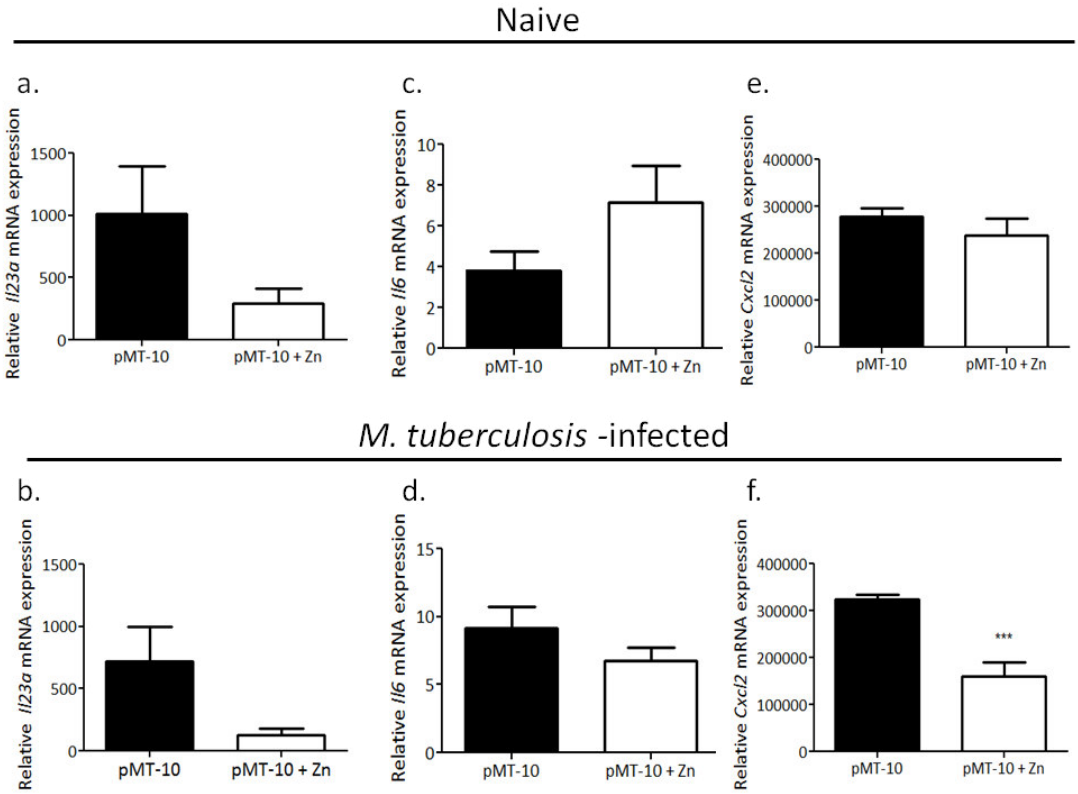

Figure 7. The low CD4$^+$ IL-17$^+$ T cells accumulation in the lungs in the presence of high IL-10 levels does not correlates with IL-23a and IL-6 expression. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (white bar) and pMT-10 control littermates (black bar) were infected with *M. tuberculosis* H37Rv as in

Figure 1. At fourteen days post-infection, RNA from uninfected mice and *M. tuberculosis*-infected mice was extracted and transcribed to cDNA, and expression of *Il23a* **(a. and b.)**, *Il6* **(c. and d.)** and *Cxcl2* **(e. and f.)** were analyzed by semi-quantitative or quantitative real-time PCR and normalized to the expression of *Ub* or *Hprt1*, respectively. Each bar shows the Mean ± SEM for 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test.

Regarding the increased of total number of IFN-γ-producing CD4 T cells in the lungs in the presence of high levels of IL-10, we questioned whether this accumulation could be related with the increased production of T cell-chemoattractants, namely CXCL9, CXCL10 and CXCL11 [173]. At the end of zinc administration, in uninfected pMT-10 mice there was an increased expression of the chemokines analysed when compared to control pMT-10 mice (Figure 8a,c and e). These differences are lost in infected mice, as the infection is already promoting the expression of these genes (Figure 8b,d and f). These data suggest that high levels of IL-10 correlates with the expression of T cell-chemoattranct genes, which likely lead to increased accumulation of IFN-γ-producing CD4 T cells.

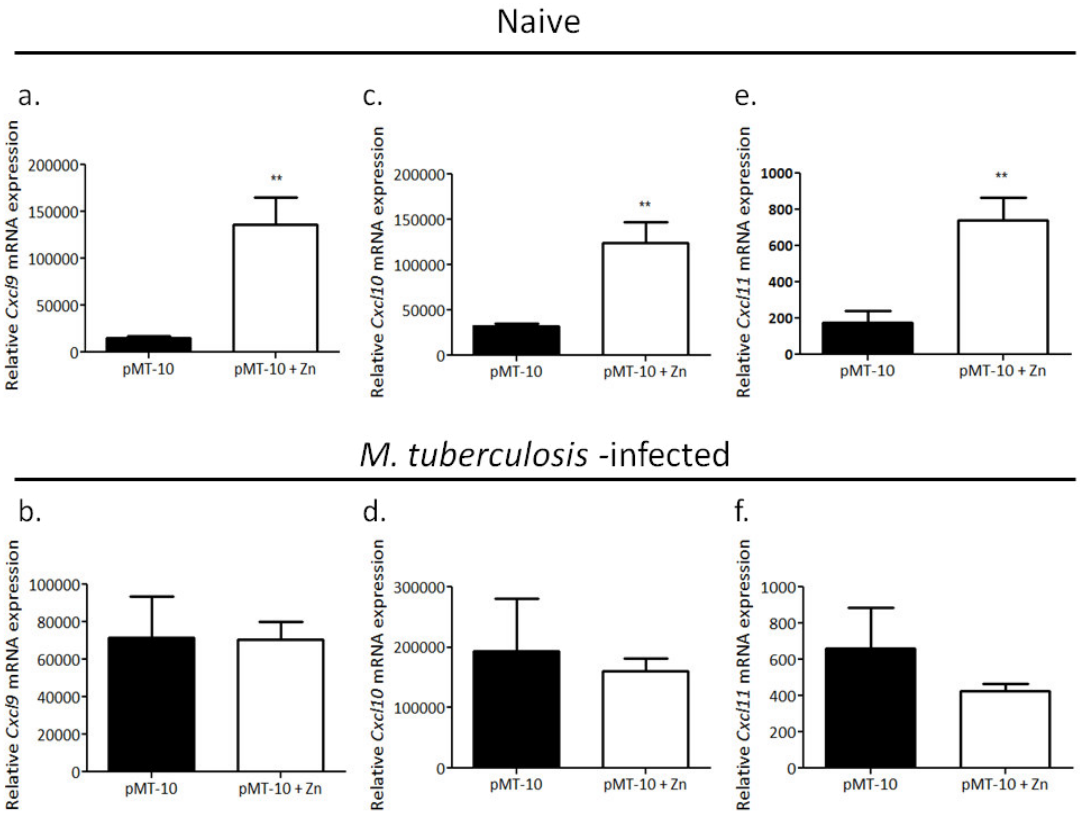

Figure 8. The high CD4⁺ IFN-γ⁺ T cells accumulation in the lungs in the presence of high IL-10 levels correlates with a T-cell chemoattrancts expression. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (white bar) and pMT-10 control littermates (black bar) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, RNA from uninfected mice and *M. tuberculosis*-infected mice was extracted

and transcribed to cDNA, and expression of *Cxcl9* **(a. and b.)**, *Cxcl10* **(c. and d.)** and *Cxcl11* **(e. and f.)** were analyzed by quantitative real-time PCR and normalized to the expression of *Hprt1*. Each bar shows the Mean ± SEM for 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (**p≤0.01).

IL-10 over-expression between day 7 and day 14 does not impact the accumulation and activation of alveolar macrophages in the lung

Taking into account the data presented above, we questioned whether the alterations in the CD4 T cell response impacted the dynamics of myeloid cell populations in the lung. As discussed before, macrophages and DCs are important players in the innate control of *M. tuberculosis* infection, and it is well established that IFN-γ produced by T cells activate these innate immune cells [174]. To address this, lung cells from *M. tuberculosis*-infected mice were stained with antibodies for CD11b, CD11c, Ly6G and MHC II. Viable cells were gated and CD11c+ and CD11b- alveolar macrophages were selected (figure 9a and b) [175, 176]. At the end of zinc administration, we observed that the total number of CD11c+ CD11b- cells in the lungs from both uninfected induced pMT-10 and control pMT-10 mice were similar (Figure 9c), as was the total number of these cells expressing MHC II (Figure 9d). In *M. tuberculosis*-infected mice, we observed that high levels of IL-10 did not impact the total number of CD11c+ CD11b- in the lung or their activation, evaluated by MHC II expression (Figure 9e and f). These data suggest that, high levels of IL-10 do not impact the accumulation and activation of alveolar macrophages in the lungs of uninfected or *M. tuberculosis*-infected mice.

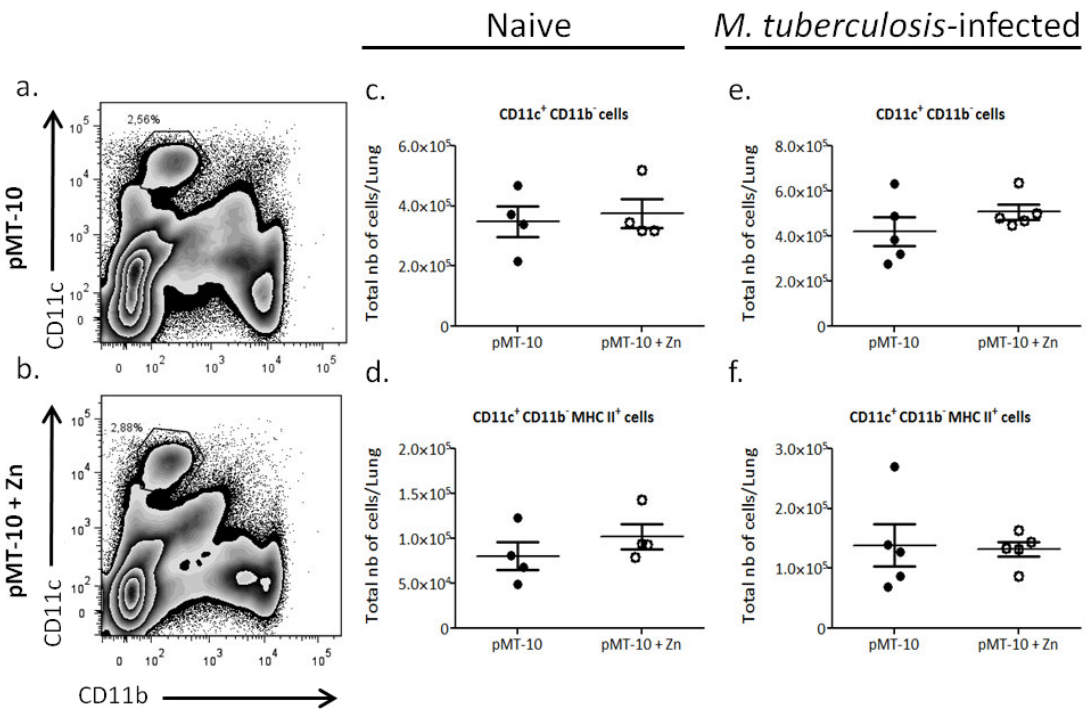

Figure 9. IL-10 over-expression between day 7 and 14 does not impact the accumulation and activation of alveolar macrophages in the lungs. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (open circles) and pMT-10 control littermates (closed circles) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, lungs from uninfected (left panels) and *M. tuberculosis*-infected mice (right panels) were prepared and stained with specific antibodies for CD11b, CD11c, MHC II and Ly6G and analyzed by flow cytometry. **(a. and b.)** The zebra plot show the electronic gate used to identify CD11c+ CD11b- cells on gated on single cells and numbers indicate percentage of CD11c+ CD11b- cells in each gate. Total number of CD11b+ CD11b- cells **(c. and e.)** CD11c+ CD11b- MHC II+ cells **(d. and f.)**. Data points show the Mean ± SEM from one representative experiment of 3 experiments with 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test.

IL-10 over-expression between day 7 and day 14 inhibits the up-regulation of MHC II molecules by DCs in the lung

DCs play an essential role in the immune response to *M. tuberculosis* infection [81]. These cells are mainly responsible for antigen presentation in the lymph nodes and subsequently triggering adaptive immunity [81]. These cells can be an important target for IL-10 since this cytokine can downregulate both MHC II expression and co-stimulatory molecules [126]. To address the impact of the high levels of IL-10 in DCs activation in the context of *M. tuberculosis* infection, lungs from *M. tuberculosis*-infected and uninfected mice were stained with antibodies for CD11b, CD11c, Ly6G and MHC II. Viable cells were gated and CD11c+ and CD11b+ DCs were selected [176].

The zebra plot show the electronic gate used to identify CD11c+ CD11b+ DCs cells gated on single cells (Figure 10a and b). At the end of zinc administration, we observed that the total number of CD11c+ CD11b+ DCs cells in the lungs from both uninfected induced and control pMT-10 mice was similar (Figure 10c). However, in the presence of high levels of IL-10, the intensity of MHC class II expression in CD11c+ CD11b+ DCs, represented by geometric mean intensity (MFI), was lower in induced than in control pMT-10 mice (Figure 10d). Similar results were obtained in *M. tuberculosis*-infected mice (Figure 10e and f). Interestingly however, the high levels of IL-10 did not impact the total number of CD11c+ CD11b+ DCs (Figure 10e). These data suggest that a high level of IL-10 does not impact the accumulation of DCs in the lungs but instead impacts their activation.

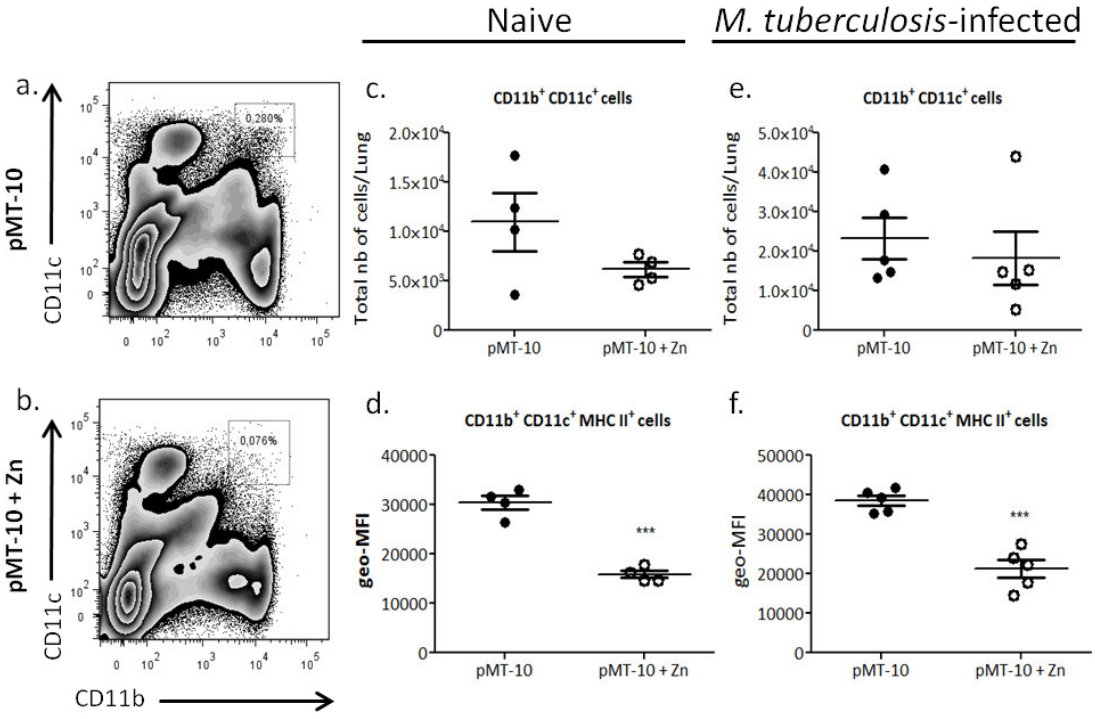

Figure 10. IL-10 over-expression between day 7 and 14 inhibits the up-regulation of MHC class II expression by DCs in the lungs. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (open circles) and pMT-10 control littermates (closed circles) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, lungs from uninfected (left panels) and *M. tuberculosis*-infected mice (right panels) were prepared and stained with specific antibodies for CD11b, CD11c, MHC II and Ly6G and analyzed by flow cytometry. **(a. and b.)** The zebra plot show the electronic gate used to identify CD11c+ CD11b+ cells gated on single cells and numbers indicate percentage of CD11c+ CD11b+ cells. Total numbers of CD11c+ CD11b+ cells **(c. and e.)** and geo-MFI of surface MHC II on $CD11b^{+}$ $CD11c^{+}$ cells **(d. and f.)**. Data points show the Mean ± SEM from one representative experiment of 3 experiments with 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (***$p \leq 0.001$).

IL-10 over-expression between day 7 and day 14 promotes the accumulation of activated inflammatory monocytes in the lungs

Monocytes are generated in the bone marrow and are constitutively released into blood circulation [175, 177]. These cells give rise to many of the macrophage and DC subsets that appear following aerosol infection with *M. tuberculosis* infection [175]. Ly6C+ monocytes or inflammatory monocytes are recruited to inflammed tissues and to secondary lymphoid organs, where in contact with differentiation signals start to differentiate in other myeloid cells [175].

We next investigated whether the presence of high levels of IL-10 impacts the accumulation and activation of inflammatory monocytes in the lungs of pMT-10 mice. To address this, lungs from induced or control pMT-10 mice were removed and stained with antibodies for CD11b, CD11c, Ly6G and MHC II. Viable cells were gated and selected for CD11b+ and CD11c- cells and, within this population, we selected SSC^{Low} and Ly6G- cells. CD11b is expressed on all myeloid lineage cells, including neutrophils. In order to exclude neutrophils, we selected Ly6G- cells (Figure 11a and b). At the end of zinc administration, we observed that in the presence of high levels of IL-10 in uninfected pMT-10 mice, the total number of CD11b+ Ly6G- SSC^{Low} cells appeared to be slightly higher compared to control pMT-10 mice (Figure 11c). Furthermore, it was interesting to observe that these cells appeared to be more activated as they express more MHC class II in the presence of high levels of IL-10 (Figure 11d). In *M. tuberculosis*-infected mice, we observed that the presence of high amounts of IL-10 induced a higher accumulation of CD11b+ Ly6G- SSC^{Low} cells (that are Ly6C+) (Figure 11e) and these cells expressed more MHC II than control pMT-10 mice (Figure 11f). These data indicate that high level of IL-10 increased the accumulation and activation of inflammatory monocytes in the lungs.

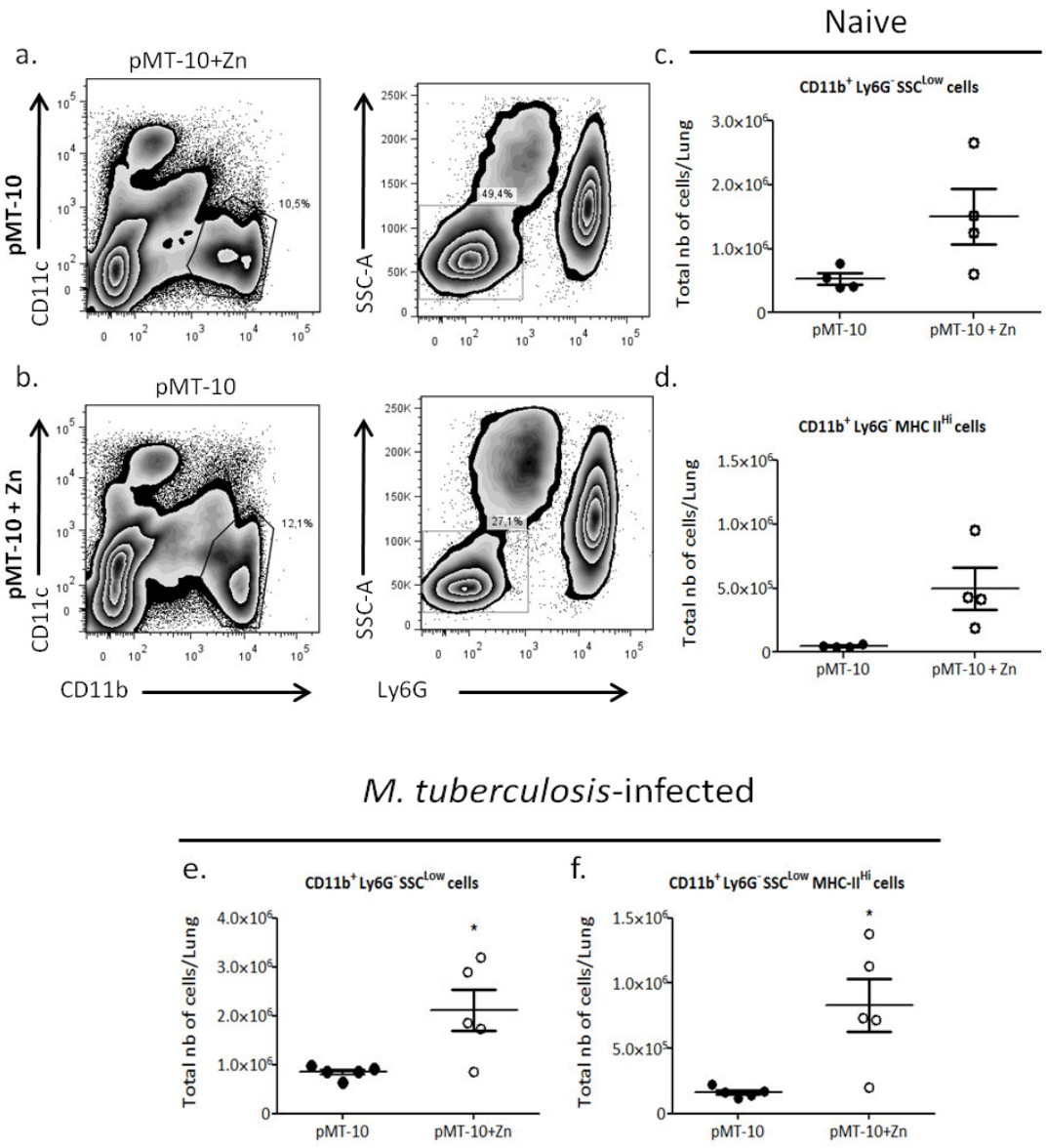

Figure 11. IL-10 over-expression between day 7 and 14 promotes the accumulation and activation of inflammatory monocytes in the lungs. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (open circles) and pMT-10 control littermates (closed circles) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, lungs from uninfected (left panels) and *M. tuberculosis*-infected mice (right panels) were prepared and stained with specific antibodies for CD11b, CD11c, MHC II and Ly6G and analyzed by flow cytometry. **(a. and b.)** The zebra plot show the electronic gate used to identify CD11b+ Ly6G- SSC^{Low} cells gated on single cells and numbers indicate percentage of cells in each gate. **(c. to f.)** Total numbers of CD11b+ Ly6G- SSC^{Low} cells **(c. and e.)** and CD11b+ Ly6G- SSC^{Low} cells MHC II^{High} **(d. and f.)**. Data points show the Mean ± SEM from one representative experiment of 3 experiments with 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (*$p<0.05$).

IL-10 over-expression between day 7 and day 14 decreases the accumulation of neutrophils in the lung

During mycobacterial infections, neutrophils are rapidly recruited to inflammed tissues, where they phagocytose the bacteria, aiding the macrophages in the control of infection. In the other hand, in established disease, neutrophils seem to contribute to pathology [178]. We questioned whether high levels of IL-10 impact the neutrophils recruitment to the lungs. To assess this, lung cells of pMT-10 mice were stained with antibodies for CD11b and Ly6G. Viable cells were gated and selected for CD11b+ and Ly6G+, a specific marker of neutrophils (Figure 12a and b). At the end of zinc administration, we observed that in the presence of high amounts of IL-10 in uninfected pMT-10 mice, the total number of CD11b+ Ly6G+ cells in the lungs was lower than in control pMT-10 mice (Figure 12c). In *M. tuberculosis*-infected mice, we verified the same results but more pronounced than in uninfected mice (Figure 12d). These data correlate with the impact of IL-10 over-expression in the IL-17 response and in the expression of CXCL2, its important chemokine for neutrophil recruitment.

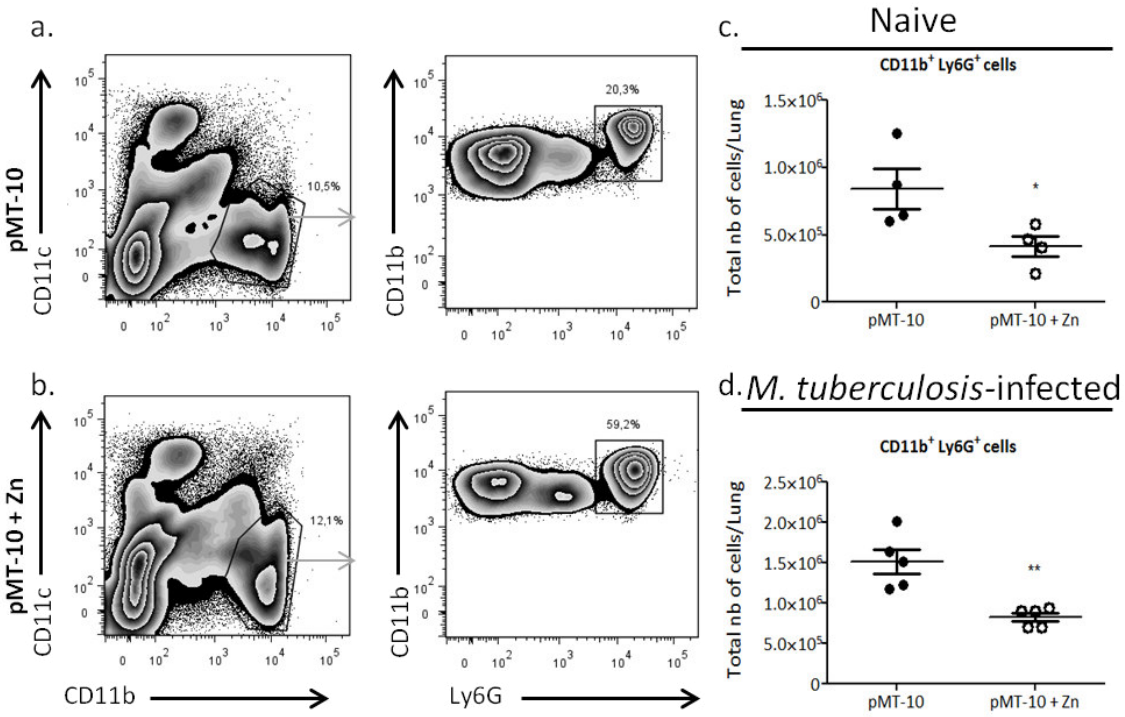

Figure 12. IL-10 over-expression between day 7 and 14 decreases the accumulation of neutophils in the lungs. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (open circles) and pMT-10 control littermates (closed circles) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, lungs from uninfected (left panels) and *M. tuberculosis*-infected mice (right panels) were prepared and stained with specific antibodies for CD11b, CD11c, MHC II and Ly6G and analyzed by flow cytometry**. (a. and b.)** The zebra plot

show the electronic gate used to identify CD11b+ Ly6G+ cells gated on CD11b cells and numbers indicate percentage of cells in each gate. **(c. and d.)** Total numbers of CD11b+ Ly6G+ cells. Data points show the Mean ± SEM from one representative experiment of 3 experiments with 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (*p<0.05).

IFN-γ blockade does not impact the accumulation of activated inflammatory monocytes in the lungs

To gain insight into the mechanisms leading to an increased total number of CD11b+ Ly6G- SSC^{Low} MHC II^{High} in the presence of IL-10, we investigated whether this was a direct consequence of IL-10 signaling or a consequence of increased IFN-γ-producing CD4 T cells in the lung (Figure 13).

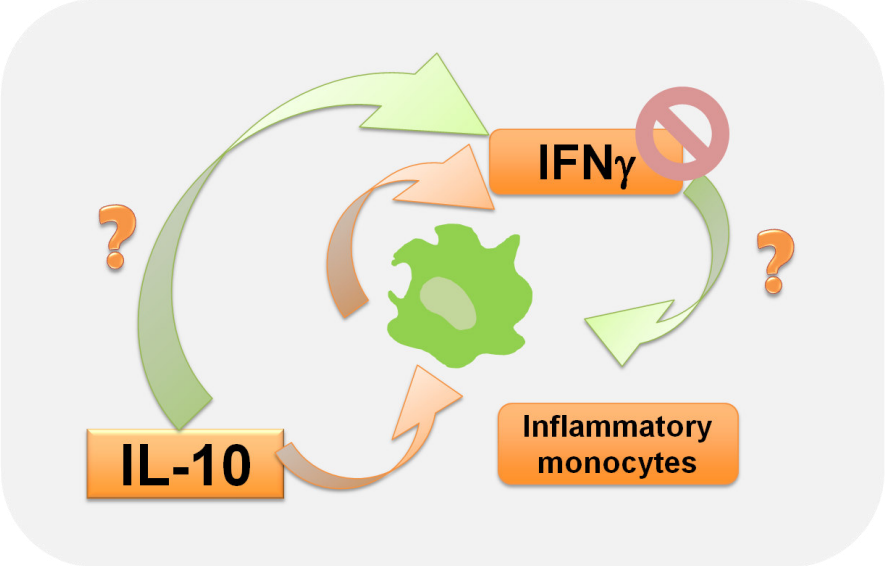

Figure 13. Schematic representation of our hypothesis about the possible mechanisms involved in increase of CD11b+ Ly6G- SSC^{Low} MHC II^{High} in the presence of high levels of IL-10, in the lungs. First hypothesis is the direct effect of IL-10 in this myeloid cell population. Second hypothesis is the indirect effect of IL-10, mediated by increased IFN-γ-producing T cells in the lung. To try answer this, we neutralize IFN-γ by administration of anti-IFN-γ.

To address the aforementioned hypothesis, we infected pMT-10 mice with *M. tuberculosis* via the aerosol route, induced the over-expression of IL-10 between day 7 and 14 post-infection, and blocked the IFN-γ signaling pathway by i.p injections of anti-IFN-γ twice a week. After the end of zinc administration, lungs from pMT-10 mice were stained with antibodies for CD11b, CD11c, Ly6G and MHC II. Viable cells were gated and selected for CD11b+ and CD11c- cells and, within this population, SSC^{Low} and Ly6G- cells were selected and the expression of MHC class II was

evaluated (Figure 14a,b and c). We observed that the blockade of IFN-γ signaling did not alter the accumulation and activation of inflammatory monocytes observed in the lung of pMT-10 mice over-expressing IL-10 (Figure 14d). These data suggest that, in the presence of high amounts of IL-10, there is an increase in the accumulation of inflammatory monocytes and in their activation, translated by increased MHC II (Figure 14) and iNOS expression (data not shown). Despite the fact of these molecules are induced by IFN-γ, in this model we observed that the IFN-γ signaling does not contribute for the activation of this population.

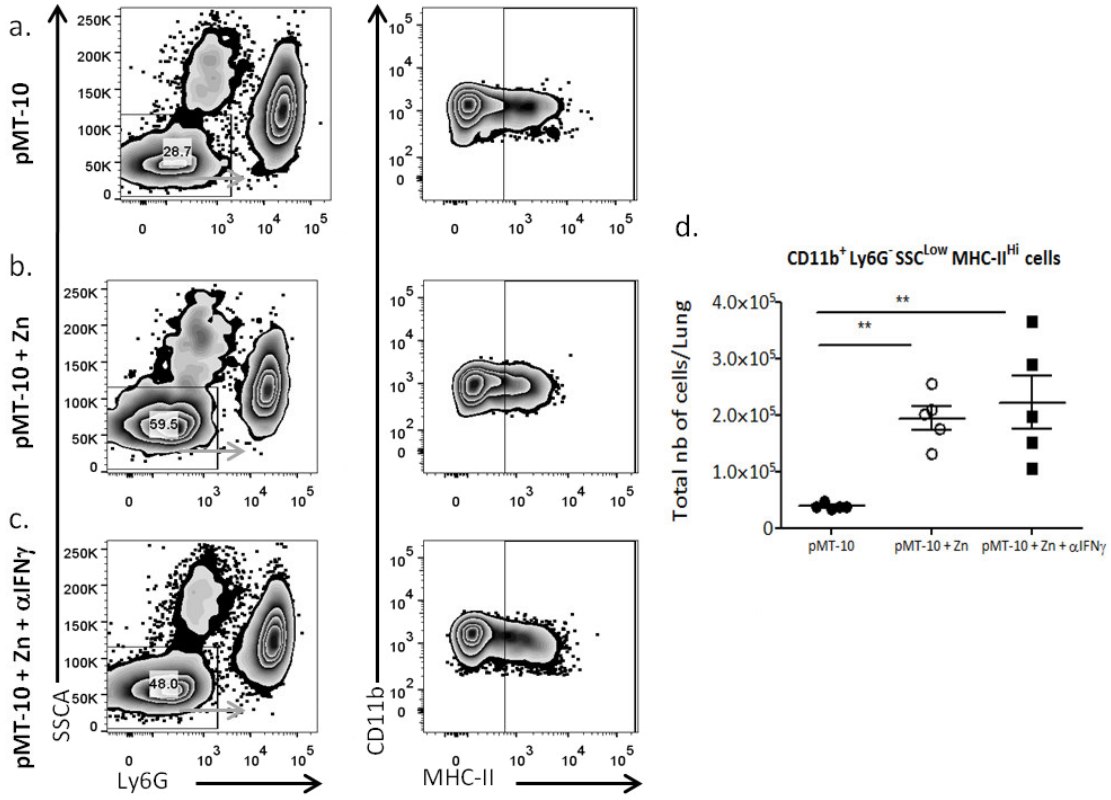

Figure 14. Neutralization of IFN-γ does not impact the IL-10-induced accumulation of activated inflammatory monocytes in the lungs. pMT-10 mice exposed to zinc between day 7 and 14 post-infection (open circles), pMT-10 mice exposed to zinc and with IFN-γ signaling blockade (squares) and pMT-10 control littermates (closed circles) were infected with *M. tuberculosis* H37Rv as in Figure 1. At fourteen days post-infection, lungs from *M. tuberculosis*-infected mice were prepared and stained with specific antibodies for CD11b, CD11c, MHC II and Ly6G and analyzed by flow cytometry**. (a. to c.)** The zebra plot show the electronic gate used to identify CD11b+ Ly6G- SSC^{Low} MHC II^{High} cells gated on CD11b cells and numbers indicate percentage of cells in each gate. **(d.)** Total numbers of CD11b+ Ly6G- SSC^{Low} MHC^{High} cells. Data points show the Mean ± SEM from one experiment with 5 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (**p≤0.01).

1.4 Effect of IL-10 over-expression in the outcome of *L. monocytogenes* infection

The innate immune response is essential for early control of *L. monocytogenes* infection [108]. After recognition, macrophages, neutrophils and Ly6C^{High} inflammatory monocytes are the main players responsible to phagocyte and kill the bacteria [108, 111]. Generally, IL-10 is accepted to have an immunosuppressive role in the protective immunity to murine listeriosis [133]. Previous reports have shown that neutralizing antibodies specific for IL-10 can transiently increase protection, but impair the clearance of *L. monocytogenes*-infected mice [150] and that IL-10 deficient mice are more resistant to infection [133]. However, other report shown opposite results [151]. To clarify these confounding results, we questioned whether transient IL-10 over-expression impairs the control of *L. monocytognes* infection, increasing the lethality associated with this experimental model. To test this hypothesis, a group of pMT-10 mice was induced to over-express IL-10 between day 2 before *L. monocytogenes* challenge and day 3 post-infection, corresponding to the pick of the protective response.

IL-10 over-expression between day 2 before L. monocytogenes challenge and day 3 post-infection increases susceptibility of mice to L. monocytogenes infection

At different time-points post intraperitoneally *L. monocytogenes* infection, two groups of infected pMT-10 mice were analysed – induced pMT-10 mice and, as a control, non-induced pMT-10 mice. The spleen and liver from pMT-10 mice were recovered and bacterial loads were compared between these 2 groups of mice. We observed that in induced pMT-10 mice there was an increase in bacterial loads in the spleen and in the liver at day 2 and 3 post-infection, compared with control pMT-10 mice (Figure 15a and b). Moreover, the induced pMT-10 mice succumbed to infection after day 3 post-infection (Figure 15c). In contrast, control pMT-10 mice controls the bacterial growth from day 3 (Figure 15a and b). These data suggest that, mice over-expressing IL-10 are more susceptible to *L. monocytogenes* infection likely as a result of a pronounced increase in bacterial burdens.

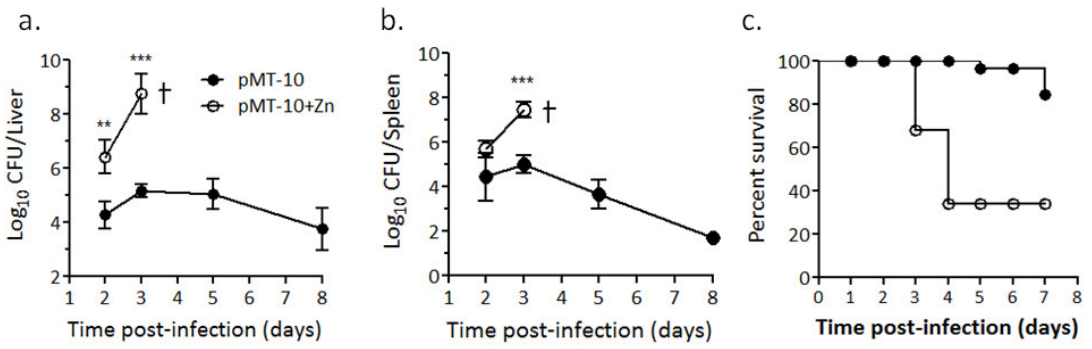

Figure 15. Mice over-expressing IL-10 are more susceptibility to *L. monocytogenes* infection. pMT-10 mice exposed to zinc between day 2 before infection and day 3 post-infection (open circles) and pMT-10 control littermates (closed circles) were intraperitoneally infected with 5x10^5 CFU of *L. monocytogenes*. **(a. and b.)** At the indicated time points post-infection, liver and spleen cell suspensions were prepared, diluted and plated into BHI medium to determine the number of viable bacteria. **(c.)** Survival curve of 12 infected mice per group. Data points show the Mean ± SEM from one representative experiment of 2 experiments of 4 mice per group. The statistical analysis between the different groups was determined by the Two-way ANOVA and the post test of Bonferroni (**p≤0.01; ***p≤0.001).

IL-10 over-expression between day 2 before L. monocytogenes challenge and day 3 post-infection impacts the accumulation and activation of Ly6C^{High} monocytes in the spleen

Recent reports have shown the Ly6C^{High} inflammatory monocytes are critical for bacterial clearance during the innate and adaptive phases of the immune response to *L. monocytogenes* infection [112]. We next investigated whether high amounts of IL-10 in induced pMT-10 mice impacted the accumulation of inflammatory monocytes and their activation in the spleen. To address this, spleen cells of pMT-10 mice were stained with antibodies for CD11b, CD11c, Ly6G, Ly6C and MHC II. Viable cells were gated and selected for CD11b+ and CD11c- cells and, within these, Ly6C^{High} cells were selected and the expression of MHC class II was evaluated (Figure 16a). We observed that, in the presence of high levels of IL-10, the accumulation of Ly6C^{High} inflammatory monocytes in the spleen was lower when compared to control pMT-10 mice (Figure 16b). Furthermore, the activation of Ly6C^{High} inflammatory monocytes, as measured by MHC class II expression, was also compromised (Figure 16c). These data suggest that, in contrast to the *M. tuberculosis* infection in the lung, the presence of high

levels of IL-10 has a detrimental impact in the accumulation and activation of Ly6C^{High} inflammatory monocytes in response to *L. monocytogenes*.

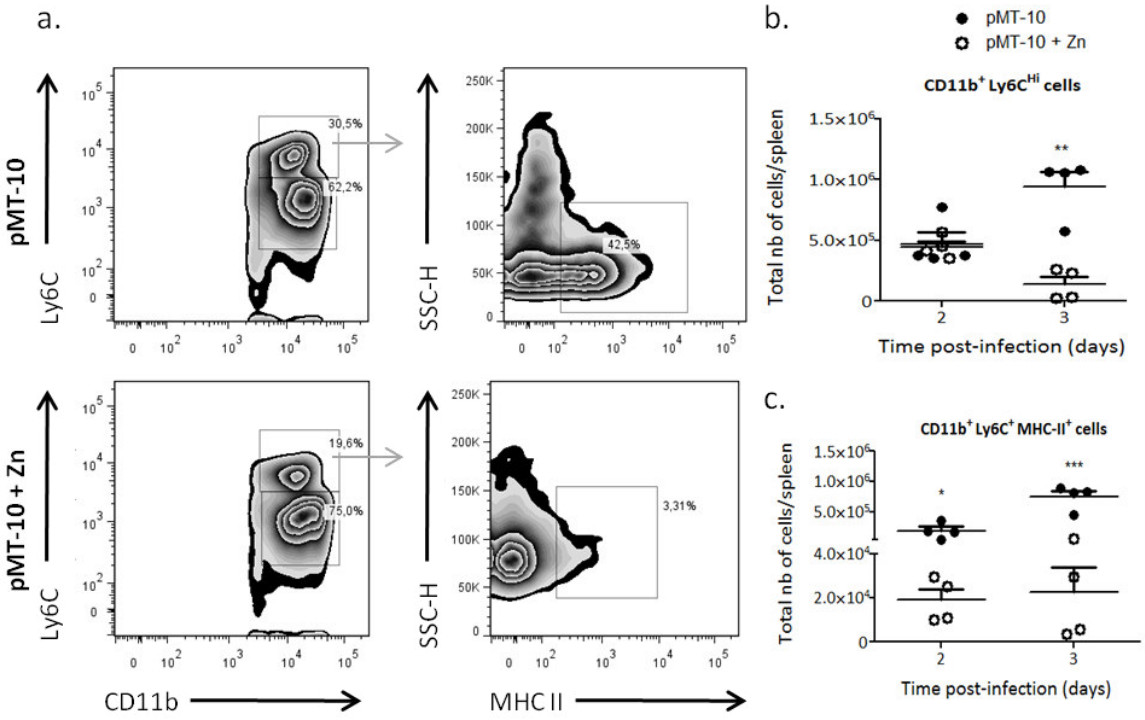

Figure 16. IL-10 over-expression during *L. monocytogenes* infection impacts the accumulation and activation of CD11b+ Ly6C^{High} MHC II+ cells. pMT-10 mice exposed to zinc between day 2 before infection and day 3 post-infection (open circles) and pMT-10 control littermates (closed circles) were intraperitoneally infected with 5x10^5 CFU of *Listeria monocytogenes.* At indicated time-points, spleen was recovered and stained with specific antibodies for CD11b, CD11c, MHC II, Ly6G and Ly6C and analyzed by flow cytometry. **(a.)** The zebra plot show the electronic gate used to identify CD11b$^+$Ly6C^{High}MHC II$^+$ on gated in CD11b cells. Numbers indicate percentage of gated cells. Total numbers of CD11b+ Ly6C+ cells **(b.)** and CD11b+ Ly6C+ MHC-II+ cells **(c.)**. Data points show the Mean ± SEM. Data from one representative experiment of 2 experiments of 4 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (*$p < 0.05$, **$p \leq 0.01$; ***$p \leq 0.001$).

RESULTS – PART II

Impact of Sirtuin2 in *M. tuberculosis* infection

2.1 Impact of SIRT2 in *in vitro M. tuberculosis* infection

SIRT2 is a histone deacetylase that regulates NF-kβ signaling through of deacetylation of the p65 subunit [163]. As a result, SIRT2 is involved in the regulation of the expression of several genes of the inflammatory response [163]. Since the studies associating SIRT2 activity with the inflammatory response are very recent, the role of SIRT2 in infection is not well understood. It has been shown that *L. monocytogenes* infection leads to SIRT2 translocation to the nucleus, leading to transcriptional repression, with impact in the control of infection [168]. Here, we addressed the role of SIRT2 in the control of *M. tuberculosis* infection.

The absence of SIRT2 in myeloid lineage does not impair the control of M. tuberculosis infection by macrophages

To gain insight into the role of SIRT2 in the macrophage response to *M. tuberculosis* infection, we used LysM-Cre+SIRT2Flox mice that are deficient in SIRT2 expression in the myeloid lineage. BMDM generated from these mice or from Cre- littermate controls were *in vitro* infected with *M. tuberculosis*. At different time-points after *M. tuberculosis* infection, BMDM were recovered and bacterial loads were compared between these 2 groups. Similar bacterial loads were observed in SIRT2 deficient mice or control littermates BMDM (Figure 17a).

Next, we investigated whether the absence of SIRT2 in BMDM impacted the production of pro- and anti-inflammatory cytokines in response to *M. tuberculosis* infection. To assess this, supernatants of infected cultures were recovered at 24 hours post-infection and the amounts of TNF-α, IL-6 and IL-10 were determined. We observed that in the absence of SIRT2 the levels of TNF-α and IL-10 were similar to those of the control BMDM (Figure 17b and d). In contrast, the amount of IL-6 secreted by *M. tuberculosis*-infected BMDM was higher when SIRT2 was not expressed (Figure 17c). Finally, we measured the levels of nitrites produced by BMDM after *M. tuberculosis* infection using the Griess method. We found that, control BMDM produce more nitrites than SIRT2 deficient BMDM (Figure 17e). These data suggest that, the absence of SIRT2 does not impact the control of *M. tuberculosis* infection by macrophages, although it likely regulates the production of some immune mediators.

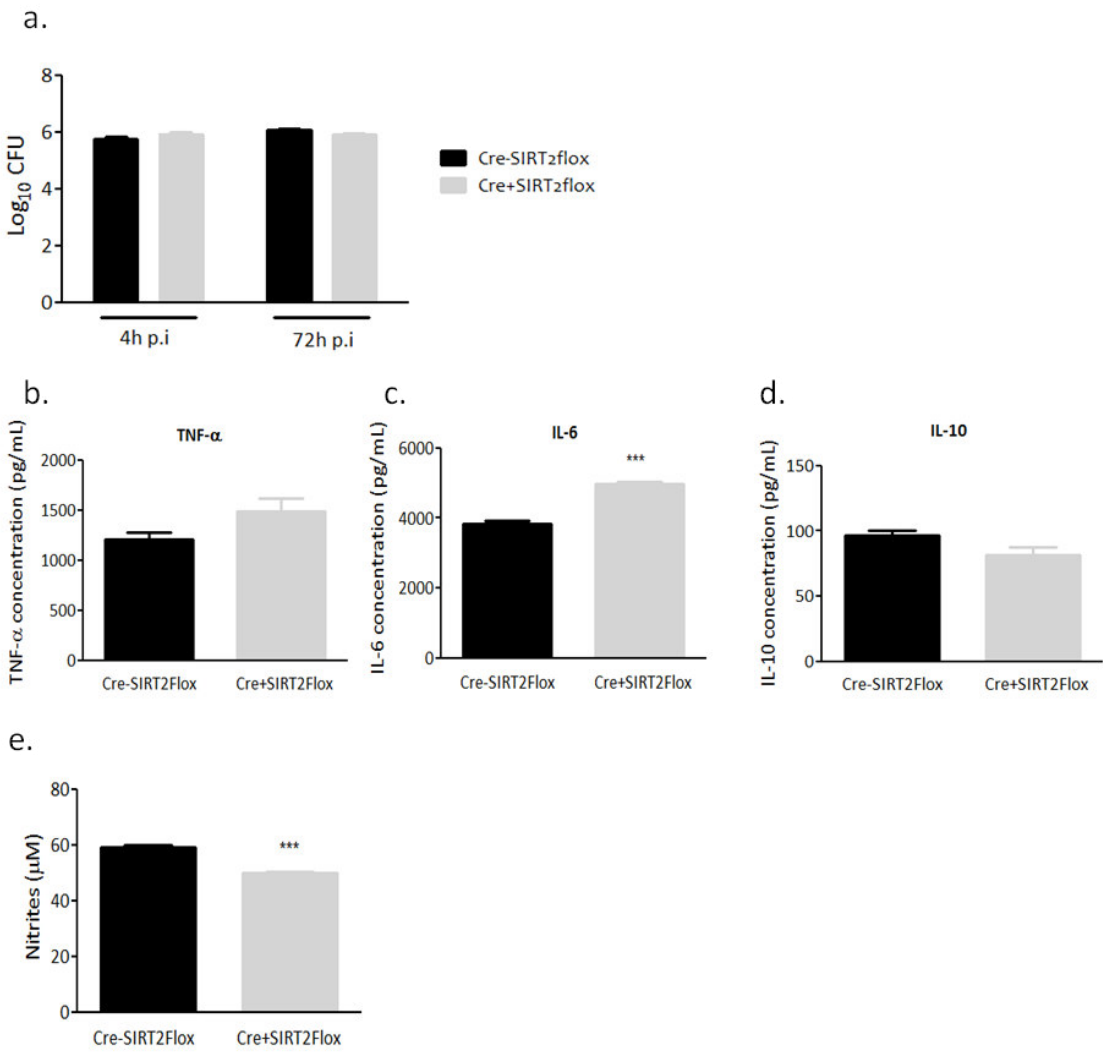

Figure 17. The activity of SIRT2 does not impact the control of *M. tuberculosis* infection by macrophages *in vitro*. (a.) Lysm-Cre+Sirt2flox+ BMDM (gray bars) and CreSirt2flox control littermates (black bars) were infected with *M. tuberculosis* H37Rv at MOI 2. At the indicated time points post-infection, macrophages were lysed and plated into Middlebrook 7H11 medium to determine the number of viable bacteria. **(b. to e.)** 24 hours post-infection, supernatants were harvested and the production of indicated cytokines and nitrites was assessed by ELISA **(b. to d.)** and Griess test **(e.)**, respectively. Each bar shows the Mean ± SEM of triplicate wells. The indicated cytokines produced by uninfected BMDMs are below detection level. The statistical analysis between the different groups was determined by the Student's t-test (***p≤0.001).

2.2 Impact of SIRT2 in *in vivo M. tuberculosis* infection

Since the complexity of interactions between different populations cannot be modeled *in vitro*, we next investigated whether the absence of SIRT2 in myeloid cells could impact the outcome of *in vivo M. tuberculosis* infection. To address this, LysM-Cre+SIRT2Flox mice and Cre- littermate controls were infected with a low dose of *M. tuberculosis* via the aerosol route.

The absence of SIRT2 in myeloid lineage impairs the M. tuberculosis dissemination to the liver

On day 30 post *M. tuberculosis* infection, lungs and liver of LysM-Cre+SIRT2Flox mice and Cre- littermate controls were recovered and the bacterial loads in these organs were determined. Interestingly, although the control of the infection in the lung at this time-point was independent of SIRT2 (Figure 18a), the bacteria burden in the liver of the infected mice was altered, as the absence of SIRT2 in myeloid cells higher bacteria burden was found (Figure 18b), indicating that SIRT2 expression in myeloid cells is required to limit *M. tuberculosis* dissemination and/or to control bacteria growth in the liver.

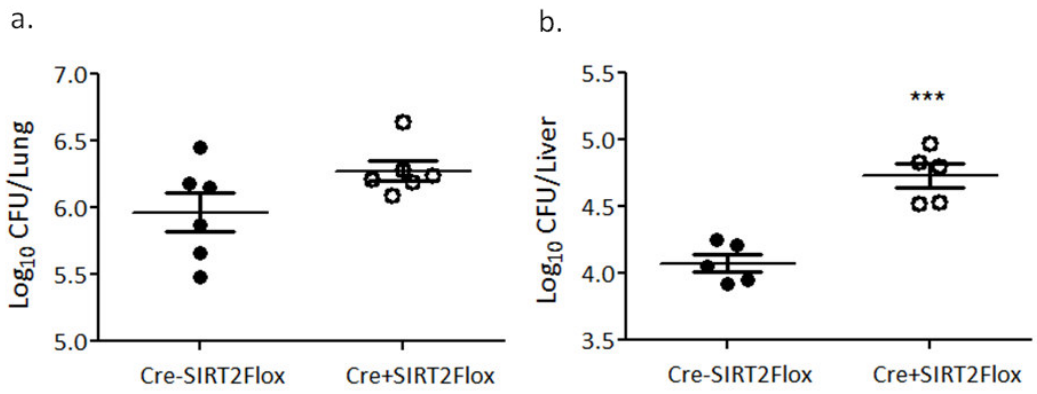

Figure 18. The absence of SIRT2 does not impact the control of *M. tuberculosis* infection but seems to favor the dissemination of bacilli to the liver. LysM-Cre+Sirt2flox mice (open circles) and CreSirt2flox control littermates (closed circles) were aerosol infected with 100 to 200 CFU of *M. tuberculosis* H37Rv. The bacterial burden on day 3 post-infection was Log_{10}(CFU)=2.00±0.03 (Mean ± SEM for five lungs). On day 30 post-infection, lung **(a.)** and liver **(b.)** cell suspensions were prepared, diluted and plated into Middlebrook 7H11 medium to determine the number of viable mycobacteria. Data points show the Mean ± SEM from one representative experiment of two experiments of 6 mice per group. The statistical analysis between the different groups was determined by the Student's t-test (***p≤0.001).

Since differences in the liver bacterial burden were observed in the absence of SIRT2 in myeloid lineage, we next evaluated the development of tissue lesions in the liver of infected mice. As shown in Figure 19, the absence of SIRT2 did not aggravate tissue lesions in the liver of infected mice when compared to control littermates at day 30.

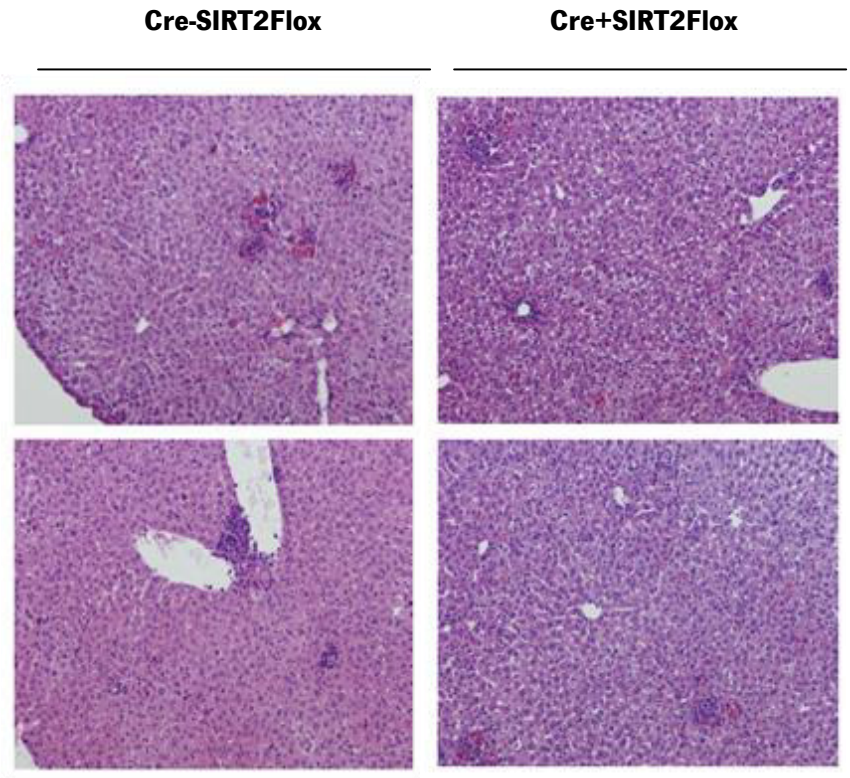

Figure 19. The absence of SIRT2 does not impact the development of tissue lesion in the liver at day 30. LysM-CreSirt2flox control littermates (left panel) and Cre+Sirt2flox mice (right panel) were aerosol infected with *M. tuberculosis* H37Rv as in Figure 18. At day 30, livers were removed, fixed in formalin, sectioned and stained with HE (original magnification x10). Data are representative of two independent experiments with six mice per group.

Altogether, our data suggests that the absence of SIRT2 in myeloid cells does not impair the control of *M. tuberculosis* infection but likely impact bacilli dissemination to the liver.

DISCUSSION

Tuberculosis is the second leading cause of death from an infectious disease remaining a serious global health problem [35]. It is well described that Th1 cell responses, in mice and in human, provide protection against to *M. tuberculosis,* but the complete elimination of the bacilli is not always achieved by the immune response [125]. If uncontrolled, the protective immune response against to *M. tuberculosis* is also involved in tissue damage [179]. Indeed, the immune response to *M. tuberculosis* needs to be tightly regulated to limit excessive inflammation that can damage the host tissues. The mechanisms mediated by host and pathogen, that generate and maintain this balance, are still poorly understood. One potential mediator of this balance is the immunosuppressive cytokine IL-10. *M. tuberculosis* induces IL-10 production by macrophages and by T cells [146, 147]. This cytokine has been shown to downregulate the pro-inflammatory response to *M. tuberculosis*, impacting IFN-γ, TNF-α and IL-12 production [121, 126]. Interestingly, although IL-10 has been indirectly implicated in chronicity and TB reactivation [180], IL-10 deficient mice show similar control of *M. tuberculosis* infection to wild-type mice [140]. Thus, the initial studies raise the question of whether IL-10 is definitely detrimental for the control of *M. tuberculosis* infection. More recently, it has been shown that the absence IL-10 is associated with increase in susceptibility of mice in later stages of *M. tuberculosis* infection [141]. In contrast, it has also been reported that the absence of IL-10 was related with increased protection to *M. tuberculosis* infection [139, 142]. Altogether, these reports clearly show the controversy that still prevails regarding the role of IL-10 in *M. tuberculosis* infection. Transgenic mice that constitutively over-express IL-10 have been an important tool to study the impact of IL-10 in mycobacterial infection. Despite the existence of several studies showing that the presence of high levels of IL-10 increases the susceptibility of mice to *M. tuberculosis* infection [143, 181], the biological mechanisms underlying this increase in susceptibility await clarification. Moreover, several players of the innate and adaptive immune response to *M. tuberculosis* are targets of IL-10, and it has been recently speculated that the timing and the source of IL-10 could differently impact the outcome of infection [125]. Taking into account these controversial reports on the role of IL-10 in *M. tuberculosis* infection we used a novel model of IL-10 over-expression, the pMT-10 mice, which over-express IL-10 under the control of a zinc-inducible promoter. This model allows the study of the impact of IL-10 over- expression in different phases of immune response, either during the onset of innate immunity to *M. tuberculosis* or during the established acquired immunity, in which the bacterial growth is controlled and maintained, or prior to T cell activation, in which DCs migrate to the lymph nodes and present *M. tuberculosis* antigens to naive T cells.

Our model thus presents several advantages as compared to previously used ones, since we can induce transient IL-10 over-expression and since the IL-10 production is not cell specific.

Our results showed that high amounts of IL-10 produced early after aerosol *M. tuberculosis* infection, namely between day 3 before challenge and day 5 post-infection, did not impact the outcome of infection. We also observed that IL-10 did not significantly impaired the T cell responses. Indeed, the high levels of IL-10 cause a decrease in T cell response in the lung only at day 20 post-infection, but the total number of T cells at day 30 is similar in both groups. Moreover, the total number of myeloid cells in the lung remained unaltered over the course of infection. This was unexpected since IL-10 is described to downregulate macrophage and DCs function. However, one possible explanation to same control of *M. tuberculosis* infection in the presence of high levels of IL-10 may be that the IFN-γ and NO are poorly induced until 2 weeks of infection and, therefore, the high amounts of IL-10 during the early innate immune response were not enough to suppress the kinetics of IFN-γ and TNF production, nor to impact DCs trafficking and T cell activation. Accordingly, it has been suggested that after aerosol *M. tuberculosis* infection, the induction of the host response is relatively slow [81] when compared with other pathogens, as in Influenza pneumonia infection and in *L. monocytogenes* infection [182]. Furthermore, Mogues *et al.,* showed that mice unable of producing IFN-γ, iNOS and MHC class II controlled the *M. tuberculosis* infection until 3 weeks post-infection, showing the these important molecules fail to impair bacterial growth until the third week of infection [183]. Therefore, it is possible during innate immune response, high IL-10 levels do not impact the *M. tuberculosis* infection, because the effector innate immune mechanisms are not yet fully activated. Other explanation to considerer, as IL-10 was over-expressed during a short time; the system was able to retrieve of immunossupressive effects of IL-10 and, subsequently did not impact the control of infection.

Recently, Joshua *et al.,* showed that in the absence of IL-10, mice were more resistant to *M. tuberculosis* infection and developed a more mature protective granuloma. Thus, IL-10 may determine the long-term outcome of TB disease [139]. Indeed, high levels of IL-10 have been found in patients with active TB [147]. In this line, we hypothesized that high levels of IL-10 during the chronic phase of infection, in which the granuloma structure matures and the bacterial growth stabilizes, may impact the outcome of infection in resistant C57BL/6 mice. We showed that after *M. tuberculosis* infection, increased amounts of IL-10 during day 50 and day 57, did

not impact the susceptibility of mice to infection. Although *M. tuberculosis* persistence in chronically infected mice is characterized by a fine equilibrium between host and pathogen, in which the replication of bacteria slow [184], our results showed that immunossupression induced by IL-10 during a short period was not enough to alter this equilibrium. This finding is interesting as it suggest that the use of IL-10 inhibitors in therapeutics for some diseases will not cause TB reactivation. This is however still complex question, once in other models of IL-10 over-expression an increase in susceptibility to infection was seen. However, in these models IL-10 is constitutively over-expressed, whereas in our model, IL-10 over-expression is transient. The potential risks of TB reactivation upon IL-10 administration is an important issue since IL-10 has been considered a potential candidate for the treatment of chronic and acute inflammation, autoimmunity and cancer [185]. A few years ago, the use of anti-TNF antibodies in patients with rheumatoid arthritis resulted in reactivation of latent TB in these patients [186, 187]. In the murine model strategies using anti-CD4 antibody [197] or inhibitors of iNOS [198] also resulted in TB reactivation. Therefore, is important that we understand the impact of novel anti-cytokine therapies in the progression of TB, since it is estimated that over 2 million people are latently infected with *M. tuberculosis*.

We also investigated the impact of IL-10 over-expression between day 7 and day 14, corresponding to the time when T cells are primed, in the outcome of *M. tuberculosis* infection. It is well established that IL-10 downregulates DC trafficking and antigen presentation [129]. We showed that induced pMT-10 mice controlled the infection in similar manner to pMT-10 mice. However, IL-10 induction during this period did impact the CD4 T cell responses. Indeed, we observed that in the presence of high levels of IL-10 there was a decrease in IL-17-producing CD4 T cells and a surprising increase in IFN-γ-producing CD4 T cells, in the lungs. Regarding the decrease in IL-17-producing CD4 T cells we considered two explanations: (i) the direct immunosuppressive effect of IL-10 in Th17 cells, and (ii) through IL-10-producing Tregs [188, 189]. It has been recently shown that IL-17- producing CD4 T cells express the IL-10 receptor α *in vivo* and that the blockade of IL-10 signaling led to an increase of Th17 cells during intestinal inflammation [188]. On the other hand, the decrease in Th17 cells in the presence of high levels of IL-10 may be indirectly mediated by IFN-γ, since that we unexpectedly observed an enhancement of these response in the presence of high levels of IL-10. Several reports have shown that the differentiation of Th17 cells is suppressed by IFN-γ *in vitro* and *in vivo [190]*. *In*

vivo, IFN-γ deficient mice exhibit enhanced Th17 cell responses in several disease models [100, 191]. Although cross-regulation of Th17 development by the IFN-γ response is well established, the underlying mechanisms are not clear [192]. Furthermore we have considered the impact of IL-10 in other molecules, such as IL-6 and IL-23a. We observed that IL-23a is slightly diminished in the presence of high levels of IL-10 being a possible explanation for the decrease in Th17 differentiation [193]. However, or data did not reach statistical significance. Concerning to IFN-γ-producing CD4 T cells, we observed an increase of these cells in the presence of high amounts of IL-10, contrary to what is described. Indeed, it is well described that IL-10 inhibits Th1 responses directly and indirectly mainly through inhibiting antigen presentation [129, 194]. However, Th1 CD4 T cells induced by mycobacterial infection do not express IL-10R [195]. One possible explanation for the observed increase in Th1 cell responses in the lung in the presence of high amounts of IL-10, may be that these cells are more activated by macrophages upon arrival to the lung, since the presence of high levels of IL-10 did not impact in the total number of IFN-γ-producing T cells in the lymph nodes. Accordingly, we observed an increase in Cxcl9, Cxcl10 and Cxcl11, important chemokines for the recruitment of IFN-γ-producing T cells. Despite the overall, and well known, immunosuppressive functions of IL-10, this cytokine is also important in the activation of NK and CD8 T cells [122, 124]. Furthermore, in human Papillomavirus, IL-10 increases CD8 cytotoxic activity but also Th1 cytokine production [196]. We will further explore the mechanisms whereby IL-10 enhances the Th1 response. Specifically, whether this is direct consequence of IL-10 or mediated by other molecules, produced by myeloid activated cells.

Next, we questioned whether the impact of high IL-10 expression in the T cell response could be impact the dynamics of myeloid cells. We observed that, at the end of zinc administration, the total number of CD11c+ CD11b- alveolar macrophages and their activation were similar in both mice groups. Furthermore, we observed that in the presence of high levels of IL-10, CD11c+ CD11b+ DCs were less activated than in control pMT-10 mice. This observation is in line with previous reports showing that IL-10 downregulates MHC class II expression [126]. In contrast, the total number of inflammatory monocytes (CD11b+ CD11c- Ly6G- SSC^{Low}) in the presence of high levels of IL-10 is higher than that observed in control pMT-10 mice. Furthermore, these inflammatory monocytes were also more activated. Indeed, this increase in total number of inflammatory monocytes that express MHC class II was unexpected. Taking into account the

increase in the Th1 response, one possible explanation is that Th1 cells when arrive to the lung activate this population. We questioned whether this increase in activated inflammatory monocytes, in the presence of high amounts of IL-10, was directly mediated by IL-10 or mediated by increase in IFN-γ-producing CD4 T cells in the lung. After IFN-γ signaling blockade, we observed in induced pMT-10 mice that the absence of IFN-γ signaling did not impact the activation of inflammatory monocytes in the lung. While this increase in MHC class II expression is new in inflammatory monocytes, but it has already described in murine splenic B cells [197]. *Tristan et al.,* showed in human primary monocytes, that the immunosuppressive effect of IL-10, were at least in part mediated by the up-regulation of the membrane associated ring-CH1 (MARCH1) E3 ubiquitin protein ligase, leading to the ubiquitination and subsequent intracellular detention of MHC class II molecules [197]. In B cells activated by IL-10, there is an increase in MHC class II expression through MARCH1 downregulation [197]. It will important to define whether MARCH1 is also downregulated in the inflammatory monocytes of the lung.

In what respects neutrophils (CD11b+ Ly6G+) we observed, that in the presence of high amounts of IL-10, the total number of neutrophils in the lung is lower than in control pMT-10 mice. We considered three possible explanations: (i) an immunosuppressive effect of IL-10 in chemokines involved in the recruitment of the neutrophils to the inflammed tissues, such as CXCL2 and CXCL8 [198]; (ii) a direct effect of IL-10 on the Th17 cells [188], as it is well described that Th17 cells can impact the recruitment of neutrophils to inflammed tissues [27]; (iii) the increased IFN-γ response may also have an impact in neutrophil accumulation. Indeed, it has been recently shown that IFN-γ-producing CD4 T cells downegulate Th17 cell responses [104] and can also impair neutrophils survival [104]. Altogether, IL-10 over-expression between day 7 and day 14 of *M. tuberculosis* infection impacted T cell response and myeloid cells activation in the lung. Despite these alterations in the cell-mediated response, the control of infection in induced pMT-10 mice was similar to that of control pMT-10 mice. In the future, it will be interesting to determine to what extent these alterations are induced directly or indirectly by IL-10 and whether or not they impact aspects of the immune response that are not required to control bacterial growth, such as transport of the bacteria to the lymph nodes and T cell priming.

The immune response to *L. monocytogenes* differs from the response to *M. tuberculosis* infection [34]. *L. monocytogenes* induces an acute infection contrary to *M. tuberculosis* that induces a chronic infection [34]. The absence of IL-10 during *L. monocytogenes* infection was shown to

increase the clearance of pathogen accompanied by stronger Th1 polarization [133]. In this regard, we decided to explore whether high levels of IL-10 during the early immune response to *L. monocytogenes* impacted the outcome of the infection. We observed that the high levels of IL-10 increased susceptibility to infection and after day 3 post-infection the induced pMT-10 mice succumb to infection (Figure 15c). We postulate that IL-10 inhibits innate immune cells functions, as these cells are crucial to control of *L. monocytogenes* infection. We observed that at day 3 post-infection, the livers of induced pMT-10 mice showed more evident signs of pathology, with more fibrosis than control pMT-10 mice. These data, led us to address the impact of IL-10 on inflammatory monocytes (CD11b+ Ly6c^{High}), since these cells were shown to be critical for the control of *L. monocytogenes* infection. We observed that, in the presence of high amounts of IL-10, the number of inflammatory monocytes was decreased in the spleen and these cells were less activated compared to control pMT-10 mice. It is possible that the increased susceptibility to *L. monocytogenes* upon IL-10 over-expression is mediated by the reduced activation of these cells, but we need further investigate this hypothesis.

It is clear that IL-10 has a different impact in *M. tuberculosis infection* and *L. monocytogenes* infection. First, *M. tuberculosis* is recognized by several innate immune cells through PRRs and is incorporated in phagossome where the live mycobacterium persists by arresting the phagolysossome biogenesis, avoiding direct antimicrobial mechanisms in macrophages; and block efficient antigen processing and presentation arresting the adaptive immune response [199]. Mycobacteria can adapt to the environment of the macrophage through mechanisms of evasion [200] causing chronic infection. On the other hand, *L. monocytogenes* escapes from phagossome to cytosol through LLO production. LLO mediates rupture of the phagossomal membrane, allowing the bacteria to access the host cytosol and rapidly grow, starting a robust cell-mediated response [110, 111]. It is well known that LLO-deficient *L. monocytogenes* strains are avirulent and fail to prime CD8 T cell response [111]. Thus, in *L. monocytogenes* infection the adaptive immunity is rapidly recruited to the inflammed tissues and the bacteria are efficiently removed. Taking into account these different mechanisms between host and pathogen it is understandable that IL-10 has a different impact in the outcome of *L. monocytogenes* and *M. tuberculosis* infection. Whereas in *M. tuberculosis* infection the effector mechanisms mediated by innate immune cells are only late activated, in the *L. monocytogenes* model the innate mechanisms are more prone to activate adaptive immunity. Thus, in *M. tuberculosis* infection the IL-10 over-expression during a short period did not impact the control of infection, while in *L.*

monocytogenes infection the early innate immune response is so robust that even during a short time, the high levels of IL-10 decrease the stability of system, increasing the mice susceptibility to infection. Furthermore, it must be taken into consideration that the *M. tuberculosis* and its host have coevolved for 70000 years, which may explain why the equilibrium between host and *M. tuberculosis* is so difficult to destabilize [201].

In second part of our work, we studied another immune modulator molecule, SIRT2, in the context of *M. tuberculosis* infection. Studies associating SIRT2 with the modulation of inflammatory responses are recent. Indeed, it has been shown that in *L. monocytogenes* infection, SIRT2 contributes to susceptibility to infection [168] but in *M. tuberculosis* infection the role of SIRT2 remains unknown. After *M. tuberculosis in vitro* infection, we observed in SIRT2 deficient BMDM a similar bacterial compared to littermate controls. In terms of cytokine production, we found higher IL-6 production, but similar IL-10 and TNF-α production. The same data were observed in *in vitro* BCG infection. Moreover, in the *in vivo* model of infection, we observed that the absence of SIRT2 did not impact the control of infection in the lung. One possible explanation for a lack of effect of SIRT2 deficiency in the control of *M. tuberculosis* infection is that this deacetylase is weakly expressed in the lung [202]. Alternatively, it is possible that more pronounced differences may be visible if a full knockout is used, rather than a myeloid specific SIRT2 KO. Interestingly, in the absence of myeloid SIRT2, *M. tuberculosis* appears to better disseminate to the liver. However, there were no visible signs of pathology in this organ. We still do not know whether, in the absence of SIRT2, the cells that contain the bacteria migrate more to the liver, or whether in the absence of SIRT2 the bacteria replicate more in the liver. One possible explanation is the expectable more inflammatory response in the lung in the absence of SIRT2, as showing in *vitro* by increase in the IL-6 levels. The increase in inflammation may be favor to the bacilli dissemination, as reported in resistant C57BL/6 in which the mycobacteria disseminate more than in the susceptible C3H mice [78]. We are studying what happens in the later stages of *M. tuberculosis* infection and when animals are infected with high doses of *M. tuberculosis,* to understand if high bacterial burden may interfere with the efficient expression of protective immunity in SIRT2 deficient mice. Moreover, we will investigate whether SIRT2 deficiency also impacts the dissemination of bacteria for other organs, such as spleen and lymph nodes.

All together, our data show that transient IL-10 over-expression differently impact chronic and acute infections and that a short period of IL-10 over-expression does not compromise the control of *M.* tuberculosis. This may be useful when the use IL-10 in therapeutics is being considered. Moreover, SIRT2, a recently described molecule involved in inflammatory response, has an impact in dissemination of *M. tuberculosis* to the liver.

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