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Impact of mycobacterial recognition by toll like receptors in the regulation of IL-10 and T helper type of responses

Impacto do reconhecimento de micobactérias por *toll like receptors* na regulação da IL-10 e nas respostas do tipo T *helper*.

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Segue o teu destino...rega as tuas plantas, ama as tuas rosas.

O resto é a sombra de árvores alheias.

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Dedico esta tese aos que são meus...

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ABSTRACT

The early immune recognition of mycobacteria is of most importance for the initiation of microbicidal mechanisms and modulation of adaptive immune responses. Macrophages and dendritic cells (DCs) are important players of the innate immune response and are among the first cells to sense mycobacteria. This initial recognition of the pathogen is only possible due to pattern recognition receptors (PRRs) on host cells, such as toll like receptors (TLRs), which activation culminates in the production of cytokines that will act as autocrine but also paracrine factors. A balanced cytokine environment is fundamental, as uncontrolled pro-inflammatory responses might culminate in tissue damage, however, an immune suppressive environment could lead to an impaired ability to control infection.

In this thesis, we addressed the impact of mycobacterial recognition on cytokine production by mouse macrophages and DCs, with an emphasis on the regulation of IL-10 expression. We have also addressed the specific contribution of TLR2 in the establishment of T helper (Th)17 type of responses upon mycobacterial infection in mice. Firstly, we compared the regulation of IL-10 by macrophages infected with two strains of *Mycobacterium tuberculosis* whose macrophage activation can be mainly mediated by TLR2 or by TLR4. We found that both strains can induce IL-10 production, however TLR2 triggering led to a rapid and strong degradation of the IL-10 mRNA, while TLR4 triggering led to the stability of IL-10 mRNA, thereby resulting in different amounts of IL-10 production. The stabilization of the IL-10 mRNA was found to be the result of the TLR4-induced activation of the TRIF/p38 pathway, thus uncovering a novel pathway for IL-10 regulation at the post-transcriptional level in the context of infection by mycobacteria. Secondly, we observed that, when compared to *M. tuberculosis*, *Mycobacterium bovis* BCG was a potent inducer of IL-10 production by DCs, which strongly contributed for the low amounts of IL-12 induced by this mycobacterium. Considering the importance of TLR2 in the recognition of both bacteria, our results also suggest that *M. bovis* BCG is a stronger activator of TLR2, when compared to *M. tuberculosis*. Thirdly, we have dissected the role of TLR2 recognition during an *in vivo M. tuberculosis* infection. We showed for the first time that TLR2 is of major importance for the maintenance of Th17 responses in the lung, and consequently for the expression of CXCL9, CXCL10 and CXCL11, chemokines implicated in protective recall responses to *M. tuberculosis*. This study provides insights into the role of TLR2 in response to *M. tuberculosis* with implications in the pathophysiology of the disease and vaccine design.

Overall, our studies provide insights on how pro and anti-inflammatory responses can be differently triggered by distinct strains and species of mycobacteria and its implications on our understanding of the host-pathogen interactions with consequences on the design of novel preventive and therapeutic strategies.

RESUMO

O reconhecimento imune precoce de micobactérias é da maior importância para a iniciação de mecanismos microbicidas e modulação de respostas imunes adaptativas. Macrófagos e células dendríticas (DCs) são células fundamentais da resposta inata e estão entre as primeiras células a reconhecer a micobactéria. Este reconhecimento inicial do agente patogénico é apenas possível graças a expressão de *pattern recognition receptors* (PRRs) nas células do hospedeiro, tais como *toll like receptors* (TLRs), cuja ativação culmina na produção de citoquinas que irão atuar como fatores autócrinos mas também parácrinos. Um perfil de produção de citoquinas equilibrado é fundamental dado que respostas pro-inflamatórias descontroladas podem resultar em dano tecidular, no entanto, uma resposta exageradamente immuno-supressora pode levar à diminuição da capacidade de controlo da infeção.

Nesta tese, estudámos o impacto do reconhecimento micobacteriano na produção de citoquinas por macrófagos e células dendríticas de ratinho, com ênfase na regulação da expressão da IL-10. Estudámos também a contribuição específica do TLR2 no estabelecimento de respostas do tipo T helper (Th)17 após infeção micobacteriana de ratinhos. Em primeiro lugar, comparámos a regulação da IL-10 em macrófagos infetados com duas estirpes de *Mycobacterium tuberculosis* em que a ativação do macrófago é maioritariamente mediada por TLR2 e por TLR4. Descobrimos que as estirpes usadas induzem a produção de IL-10, no entanto, a ativação por TLR2 levou a uma rápida e acentuada degradação do mRNA da IL-10, ao passo que a ativação por TLR4 levou à estabilização do mRNA da IL-10, resultando assim em diferentes quantidades de IL-10 produzida. A estabilização do mRNA da IL-10 resultou da ativação da via TRIF/p38 em resposta a sinais TLR4, revelando assim uma nova via de regulação da IL-10 ao nível pós-transcripcional no contexto de infeções por micobactérias. Em segundo lugar, observámos que, em comparação com *M. tuberculosis*, *Mycobacterium bovis* BCG foi um potente indutor da produção de IL-10 por DCs, o que contribuiu em muito para os baixos níveis de IL-12 induzidos por esta micobactéria. Considerando a importância do reconhecimento de ambas as micobactérias por TLR2, os nossos resultados sugerem também que o *M. bovis* BCG é um potente ativador do TLR2, quando comparado com o *M. tuberculosis*. Em terceiro lugar, analisámos o papel do reconhecimento via TLR2 durante a infeção *in vivo* com *M. tuberculosis*. Mostrámos pela primeira vez o TLR2 é da máxima importância para a manutenção das células Th17 no pulmão e, consequentemente, para a expressão de CXCL9, CXCL10 e CXCL11, quemoquinas envolvidas em respostas protetoras de memória contra *M.*

tuberculosis. Este estudo dá a conhecer um novo papel do TLR2 na resposta ao *M. tuberculosis* tendo implicações a nível da pato-fisiologia da doença e desenho de vacinas.

Em suma, nossos estudos fornecem informação sobre como as respostas pró e anti-inflamatórias podem ser desencadeadas por diferentes estirpes e espécies micobacterianas, bem como possíveis implicações no entendimento das interações agente patogénico-hospedeiro, com consequências no desenho de novas estratégias preventivas e terapêuticas.

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ABBREVIATIONS LIST

APC	Antigen Presenting cell
ARE	AU-rich elements
BCG	Bacillus Calmette-Guérin
CD	Cluster of differentiation
CpG	Cytosine phosphodiester guanine
CR	Complement receptor
CTL	Cytotoxic T cell lymphocyte
DCS-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DLN	Draining lymph node
DUSP	Dual specificity protein phosphatase
ERK	Extracellular-signal-regulated kinases
ICAM	Intercellular adhesion molecules
IFN	Interferon
IL	Interleukin
iNOS	inducible Nitric oxide synthases
LAM	Lipoarabinomannan
JNK	c-Jun N-terminal kinases
LM	Lipomannan
LPS	Lipopolysaccharides
Ltbi	Latent tuberculosis infection
Man-Lam	Mannosylated LAM
MAPK	Mitogen activated protein kinase
MDR	Multidrug resistant
MyD88	Myeloid differentiation primary response gene 88
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOD	Nucleotide-binding oligomerization domain-containing protein
Pam3Csk	(S)-[2,3-*Bis*(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys$_4$-OH . 3HCl
PAMP	Pathogen-associated molecular patterns
PIM	Phosphatidylmyo-inositol Mannoside
PRR	Pattern recognition receptors
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROI	Reactive oxigen intermediates
Th	T helper cell
TICAM	TIR domain-containing adapter molecule
TIR	Toll/interleukin-1 receptor
TIRAP	toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll like receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTP	Tristetraprolin
UTR	Untranslated region
WHO	World Health Organization
XDR	Extreme drug resistant

THESIS PLANNING

The present thesis is organized in 6 different chapters.

In chapter 1 of the thesis, a general introduction is presented. Briefly, in this chapter is provided a summary of the literature on the clinical features of tuberculosis, epidemiology of the disease, prevention and treatment. The genetic basis for *M. tuberculosis* is presented, followed by the description the host-mycobacteria interactions using the mouse experimental model as a reference. Finally, chapter 1 ends with an overview of IL-10 in the immune response in particular to mycobacteria and mechanisms of regulation of this cytokine.

In chapter 2, the aims of the present dissertation are proposed.

Chapter 3 is subdivided into 3 parts and comprises the experimental results. In chapter 3.1, the study "*TLR2 and TLR4 signals differently regulate IL-10 expression by macrophages at the post-transcriptional level*" is presented. Within this study we show that TLR2 and TLR4 differential triggering results in different levels of post-transcriptional regulation of IL-10 thereby leading to the secretion of different amounts of this cytokine by macrophages. In chapter 3.2, the study "*Higher IL-10 and lower IL-12 production by dendritic cells are induced upon stimulation with Mycobacterium bovis BCG as compared to Mycobacterium tuberculosis.*" is presented. This work shows that infection with *M. bovis* BCG, as compared to *M. tuberculosis*, leads to a stronger IL-10 production by dendritic cells. This IL-10 production is mainly mediated by TLR2, having detrimental implications in the production of IL-12. In chapter 3.3, the work "*TLR2 deficiency by compromising p19 (IL-23) expression limits Th 17 cell responses to Mycobacterium tuberculosis*" is presented. This study demonstrates that upon *M. tuberculosis* infection, TLR2 signals, although not needed for the generation of Th17 cells in the draining lymph nodes, are needed for the maintenance of these cells in the lung due to its involvement in the regulation of p19 (IL-23) expression, a survival factor for Th17 cells.

The general discussion of the experimental work is presented in chapter 4.

CHAPTER 1

INTRODUCTION

Tuberculosis has plagued humankind for several centuries and still nowadays this disease prevails as one of the deadliest induced by an infectious agent (1).The causative etiological agent of this malady is primarily *Mycobacterium tuberculosis* although other mycobacteria from the *M. tuberculosis* complex can also induce disease (1). Tuberculosis is a highly contagious sickness since it spread through the air. When a person with the active disease coughs, sneezes or spits the mycobacteria is propelled into the air being eventually inhaled by people nearby (2). Thus, tuberculosis is primarily a disease of the lung, however, on some occasions this disease can be extrapulmonary as it is the case of immune-compromised individuals (3).

1.1 Mycobacteria and the *Mycobacterium tuberculosis* Complex

The *Mycobacterium* genus comprises over 100 closely related species of mycobacteria as concluded after comparing their 16S rRNA sequences (4). The majority of those species are non-pathogenic mycobacteria and environmental mycobacteria. In addition to *M. tuberculosis,* examples among the pathogenic species are *Mycobacterium leprae* and *Mycobacterium ulcerans*, the causative agents of Leprosy and Buruli ulcer, respectively.

Essentially, mycobacteria are irregular rods of 0.3–0.5 mm in diameter with variable length (5), non spore-forming, aerobic bacteria that by virtue of lacking the outer cell membrane are considered to fit into the Gram positive bacteria category. Yet, mycobacteria are non-canonical Gram positive bacteria since they fail to stain with crystal violet (4, 6). Additionally, mycobacteria are typically acid-alcohol fast staining bacteria (4, 6). These typical staining features are due to the unique composition of mycobacteria cell wall that is characterized by a high content of mycolic acids making up more than 50% of the mycobacteria dry weight (7).

Typically, the cell wall composition of mycobacteria, namely of *M. tuberculosis*, consists of: i) an external portion that is mainly composed by a thick layer of mycolic acids; ii) next to the mycolic acids, facing the external side, are components such as mannose-containing biomolecules such as mannose-capped lipoarabinomannan (Man-LAM), the related lipomannan (LM) and manno-glycoproteins, that are common to all pathogenic mycobacteria and are not present on fast-growing mycobacteria which are considerably less pathogenic; and iii) an internal layer composed mainly by arabinogalactan, phosphalidyl-*myo*-inositol mannosides (PIMs), and peptidoglycans (7). Furthermore, the thick layer of mycolic acids is responsible for the impaired uptake of nutrients resulting in the slow growth of mycobacteria, as is the case of *M.*

tuberculosis, and is also responsible for mycobacterial resistance to degradation via lysossomal enzymes (7).

The classical *M. tuberculosis* complex (MTC) integrates typically intracellular mycobacteria species that are known to induce tuberculosis in different hosts: *M. tuberculosis*, although the responsible for the majority of human pulmonary tuberculosis cases, as mentioned before, can also induce extrapulmonary diseases such as meningitis; *Mycobacterium africanum*, an agent of human tuberculosis in sub-Saharan Africa; *Mycobacterium microti*, the cause of tuberculosis in voles and recently found to be more frequent as a human tuberculosis causing agent than initially thought; *Mycobacterium bovis*, which afflicts a panoply of mammalians including humans and that prior to pasteurization protocols was a major cause of human tuberculosis; and *M. bovis* bacillus Calmette-Guérin (BCG), the attenuated version of *M. bovis*, currently used as the vaccine against tuberculosis (8-15). This complex also includes *Mycobacterium caprae*, that primarily afflicts goats and in rare cases humans (16, 17) and *Mycobacterium pinnipedii*, that primarily infects seals (18). *Mycobacterium canettii* is also commonly accepted as a member of this group and gathers the oryx bacillus and the dassie bacillus being very rarely encountered and infrequently causing disease in humans (19-21).

As mentioned above, although *M. tuberculosis* has been identified as the most common bacteria responsible for inducing tuberculosis worldwide, *M. africanum* and *M. bovis* share with *M. tuberculosis* some burden regarding tuberculosis development in certain parts of the globe (22), inducing similar pathology and course of infection (23).

M. tuberculosis in particular, is a slow-growing microbe that under favorable laboratory conditions divides every 12 to 24 hours (6). This low dividing rate is suggested to account for the classically sub-acute to chronic development of the disease and also to the long time necessary to achieve visible growth *in vitro* (6). Furthermore, *M. tuberculosis* is also a facultative intracellular bacterium that is able to divide inside macrophages and other mammalian cells (9). H37Rv is one of the most well characterized *M. tuberculosis* strains and widely used in research. Its genome has been completely sequenced in 1998 (24) and since then this type of information has opened ground to identify genes that translate into virulence factors or antigens, to which the host immune system responds, and also to identify putative target genes used in chemotherapy.

The *M. tuberculosis* H37Rv genome has approximately 4 Mbp and encloses around 4000 genes (24). It differs, in a large extent, from other bacteria since a great deal of its coding power is dedicated to the production of enzymes implicated in lipogenesis and lipolysis as well as

in two families of glycine-rich proteins that might correspond to a source of antigenic variation (24). A function has been assigned to 52% of the 3995 predicted proteins and 376 of putative proteins share no homology with known proteins hence being possibly exclusive to *M. tuberculosis* (25).

Having these findings into consideration and given the growing data on the mycobacterial field, the knowledge on how certain genes may interfere with basic functions of the pathogen is of major interest. This is true from the drug design point of view as well as to further understand how the interaction between mycobacteria and host takes place.

1.2 Epidemiology

According to the health survey reported by the World Health Organization (WHO), in 2009 there were approximately 9.4 million incident cases of tuberculosis, most of which occurred in Asia (55%) and Africa (30%). Smaller proportions of new incident cases occurred in the Eastern Mediterranean Region (7%), the European Region (4%) and in the Americas (3%). More specifically, in 2009 the five countries with the highest rate of incident cases were India (1.6–2.4 million), China (1.1–1.5 million), South Africa (0.40–0.59 million), Nigeria (0.37–0.55 million) and Indonesia (0.35–0.52 million). India by itself accounts for one fifth (21%) of all tuberculosis cases worldwide and combined with China these two countries account for 35% of tuberculosis incidence (26).

It is estimated that one third of the world´s population is latently infected and, of those, 5% to 10% will develop clinical signs of primary infection within two years post exposure (27). Once in contact with mycobacteria, a failure of the immune system leads to the development of the disease for which the clinical symptoms are in most cases cough (sometimes with blood), fatigue, weight loss and fever (2, 28). Nevertheless, tuberculosis hardly develops from a primary infection as the immune system manages to promote dormancy of the mycobacteria, stage known as Latent tuberculosis infection, where host and mycobacteria seem to find a balance in which there are neither clinical symptoms nor clearance of the mycobacteria (23, 27, 29). Yet, mostly due to host factors, if such balance is broken, reactivation can occur allowing for tuberculosis to manifest (1, 27). The most common factors associated with the development of active tuberculosis, either due to reactivation or to a primary exposure to mycobacteria, are: i) a lifestyle under unsanitary and poor nutritional conditions; ii) human immunodeficiency virus

(HIV)/tuberculosis co-infection; iv) diabetes; and v) chemotherapy, such as anti-TNF therapy for rheumatoid arthritis (28).

Portugal has been considered the fifth country in Europe with the higher case rates, right after Estonia, Bulgaria, Lithuania and Romania (30). However, more recently, it was estimated that, as compared to 2009, the number of new incident cases of tuberculosis in Portugal decreased 11% in 2010 with an incidence rate of $22,3/10^{5}$ (a total of 2372 new cases) (31).

1.3 Prevention and treatment

For several years many research studies have devoted their efforts into different preventive vaccination approaches with no major satisfactory results usable for the tuberculosis containment problem. So far, the only licensed vaccine against tuberculosis is still *M. bovis* BCG (32). Since its discovery more than 3 billion people have been vaccinated making of BCG the most used vaccine worldwide (33).

M. bovis BCG is a live isolate of *M. bovis* which was attenuated through numerous *in vitro* passages carried out by Albert Calmette and Camille Guérin and was firstly tested in humans in 1921 (34, 35). Early (34) and modern (36-39) trials have demonstrated that BCG is highly efficient in limiting disseminated childhood tuberculosis. In fact, an epidemiological meta-analysis study estimates that in the year of 2002, among the 100, 5 million BCG administrations given to children, 29 729 cases of tuberculous meningitis and 11 486 new cases of miliary tuberculosis were prevented (39). However, concerning adult tuberculosis, BCG efficacy is less obvious and seems to offer a variable range of protection (0–80%) (40, 41) indicating that BCG is not the ideal protective vaccine against adult tuberculosis. So far, a specific reason as to why BCG vaccination leads to such disparity in protection has not been identified; this seems to be a multi-factorial and complex scenario. For instance, before the original strain of BCG (Pasteur, at Institute Pasteur) was lost, it was distributed among several laboratories (42, 43), so a massive production of this vaccine by different producers began generating different BCG vaccines that diverge in their genetic background and antigenic composition (43-45), most likely leading to different host immunization responses; also, the fact that BCG vaccinated individuals might have been previously exposed to environmental mycobacteria (46, 47) in areas where these are endemic (47-49) could also contribute for BCG protection variability; as well, differences in the route of administration, age when the administration occurs and method of vaccine preparation could also add for the inconsistency at the protection level achieved with BCG vaccination against adult

tuberculosis (43, 50, 51). The host´s immunogenetic background maybe as well an important aspect during vaccination and infection itself. Accordingly, susceptibility to mycobacteria, as well as BCG efficacy, have been associated to certain host genetic features such as specific HLA-haplotypes, interferon-γ (IFN-γ) polymorphisms and the *SLC11a1* gene, in the past known as *Nramp1* (42, 52). Along with the variable protection achieved against pulmonary tuberculosis, BCG seems to have the inability to induce life-long protection. Indeed, there are some studies suggesting that BCG affords protection only during a period of 10 to 20 years, time when adult tuberculosis becomes a serious risk (53-55), raising the question as to whether a second immunization (boost) during an individual lifetime should be administered so that protection is maintained (56). The usage of BCG as an immunization boost is not recommend by the WHO (57) and there is also evidence that repeated antigen (58-60) or BCG (58, 60, 61) exposure might pose serious problems such as severe lung tissue damage.

Ideally, a vaccination protocol better than BCG, in terms of efficacy and safety, should be able to prevent disease in both healthy and HIV infected patients inducing protection not only in newborns but also in adults. However, this might prove rather difficult to achieve in just one type of vaccine, therefore more studies contemplating basic research together with clinical trials are needed.

Two major groups of vaccine candidates have been proposed in the last years: live recombinant (r) BCG, (r) *M. tuberculosis*, or other genetically modified mycobacteria, conceived to replace BCG and booster (sub-unit) vaccines aimed to improve BCG efficacy (42, 51). Given the impairment of BCG vaccination in reaching a good and lifelong level of protection against tuberculosis, new strategies have been put forward. It is interesting that although BCG is not the ideal vaccine due to the aspects mentioned before, the majority of vaccine candidates in clinical trials consist in strategies based on manipulating the immune response to BCG, either by recombining it with further mycobacterial antigens or by boosting it with those same antigens. It seems that years of research were not enough to find a perfect BCG-independent vaccine thus making of this old and only approved vaccine against *M. tuberculosis*, a central and compelling player on immunization. Therefore, more studies are necessary to further understand the immune response driven by BCG, so that new immune targets are identified for vaccine design.

Once diagnosed with tuberculosis, the patient should undergo, uninterruptedly for six to eight months, a full course of anti-tuberculosis drugs that are, more commonly, isoniazid and rifampicin (62).

A factor accountable for the augmented incidence of new tuberculosis cases is the appearance of multidrug resistant (MDR–tuberculosis) strains and, more recently, extreme drug resistant strains (XDR–tuberculosis). Essentially, MDR-tuberculosis requires longer periods of treatment with second-line drugs which becomes more expensive and also involves more side-effects. Furthermore, XDR-tuberculosis are resistant to first- and second-line drugs which poses a serious problem regarding the finding and application of an effective treatment thus being imperative that tuberculosis control is managed accurately (63-65). Drug resistance occurs mainly in areas with poor tuberculosis control programmes, where an improper drug treatment administration regimen might take place (65), however, even in developed locations if there is no correct compliance of the treatment, drug resistance can occur. A global trend survey has identified Eastern Europe (specially Estonia, Latvia and Russia), China and Iran as problematic regions for the increase of drug resistant tuberculosis cases but further and deeper surveys are in place to accurately obtain a global scenario of these MDR/XDR-tuberculosis cases (66).

1.4 Host and Pathogen: the immune response to *M. tuberculosis*

Inhalation of microscopic droplets with few *M. tuberculosis* bacilli is believed to be sufficient to cause tuberculosis infection. Approximately 90% of the individuals that contact with mycobacteria become infected and although not being capable of eliminating the pathogen, develop an immune response that is able to contain infection (28). Upon infection with mycobacteria, the pathogen is contained in well organized polymorphic cellular structures, called granuloma, with no further tissue damage to the host (67, 68). This type of lesion is encapsulated and will remain as calcified scars (67-69). In this stage, mycobacteria, although turning their metabolism dormant enough so no multiplication occurs, manage to keep silently viable for long periods of time (67, 68, 70, 71). This is the archetypal apparent win-win situation in the battle host *versus* invading mycobacteria and is commonly known as the asymptomatic stage or latency. Yet, so this level of protection is maintained, the immune response needs to be tightly regulated otherwise resulting into a harmful outcome culminating into disease reactivation. Once this balance is broken, due to, for example, an immune suppressive state, the individual no longer controls mycobacterial dormancy and will develop signs of the disease (67-69). Among the

people that become infected, only 5%-10% will develop active tuberculosis after primary infection (2, 28). In this case, the host immune response is not skilled to contain mycobacterial growth and due to unregulated cellular events, host tissue damage will occur and bacterial burden will rise. Accordingly, as intense inflammation continues, granuloma will get enlarged and caseous necrotic and cavitary lesions will develop (67, 68).

The immune response to mycobacteria is classically divided into 2 types, the innate immune response and the adaptive immune response (9, 72, 73). Both types of response, by secreting several immune factors, inducing cytokines and chemokines, shape the immune response to *M. tuberculosis* (9, 72, 73). Cytokines are classified as peptides, proteins or glycoproteins that operate in an autocrine and/or paracrine manner and typically act in a network as immunomodulatory agents thus providing pro- or anti- inflammatory signals that outline the cellular immune responses (74, 75). Some of these cytokines like TNF, IFN-γ, IL-10 (all produced by cells from both the innate and adaptive immune responses), IL-23 and IL-12 (both only produced by innate cells) are important factors not only to amplify and regulate the innate immune response but also to determine the generation of the adaptive immune response (73, 74). Chemokines, on the other hand, are a subtype of cytokines that, in the case of mycobacterial challenges, signals into inflammatory cells rendering them the ability to migrate to their proper action places (72, 74).

Once mycobacteria are deposited in the lung, the innate players become activated thereby initiating their own microbicidal plan (9, 76, 77). Although mycobacteria are facultative intracellular pathogens, they are very often found inside different cell types (78-88). The cell infection process occurs through phagocytosis, a dynamic process by which mycobacteria are uptaken by the innate cells (89) and that is initiated by several receptors on the cell surface. The family of phagocytic receptors is large and includes: mannose receptors, mannose binding lectin, class A scavenger receptors, complement receptors (CR; CR1, CR3, CR4), surfactant protein A and DC-specific intracellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN) (89, 90). Post-phagocytic events will lead to partial microbe degradation and further exposure of antigens at the cell surface via the Major Histocompability Complexes (MHCII and MHCI) ultimately leading to the activation of adaptive immune CD4+Th cells and CD8+T cells (91).

1.4.1 Mycobacterial recognition

In order that the immune events mentioned above happen efficiently, ranging from the innate activation to adaptive driving responses, a very early event needs to take place: the invading pathogen has to be sensed by the innate immune system. Microbes, due to components at their surface or due to secreted compounds, both types generally known as Pathogen-associated molecular patterns (PAMPs), will be recognized by different types of cells. This recognition is only possible owing to the existence of several specific receptors, known as Pattern-recognition receptors (PRRs) (92-95), localized either at the surface or inside host cells (95-97). The triggering of PRRs by PAMPs is fundamental for the activation of microbicidal mechanisms, cytokine production and initiation of adaptive immunity by cells of the innate arm of immunity (98-100). Some examples of PRRs are toll like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) receptors, DC-SIGN, and dectin-1 (C-type lectin receptors) (99, 101) and all of them have been shown to be involved in the recognition of *M. tuberculosis* (72, 94, 102).

1.4.1.1 Toll like receptors

Among all PRRs, TLRs have been vastly studied and identified either on the cell surface or inside cells in endosomes, and their cytokine profile induction has been vastly characterized (95, 98, 99, 103, 104). Until now, 10 human and 12 mouse TLRs were identified with TLR1-TLR9 highly conserved in both species (105).

TLRs recognize a plethora of mycobacterial molecular patterns and TLR2, -4 and -9 are the best characterized TLRs in the recognition of *M. tuberculosis* (7, 94, 106). Briefly, examples of mycobacterial ligands recognized by these TLRs are listed below: i) TLR2 hetero-dimerizes either with TLR1 or TLR6 and these complexes recognize molecules like LAM, LM, PIM, 19kDa glycoprotein (TRL2/TLR1) and dyacylated lipoproteins (TLR2/TLR6); ii) TLR4 is responsible for the recognition of molecular patterns such as PIMs, heat-shock proteins 60/65 and 70, this last one also recognized by TLR2; and iii) TLR9 recognizes unmethylated cytosine-phosphate-guanine (CpG) motifs in mycobacterial DNA (7, 72, 94, 106-108).

Mycobacterial recognition via different TLRs initiates several intracellular signaling cascades that culminate with the production of important pro- and anti- inflammatory cytokines involved in the host immune response to mycobacteria such as TNF, IL-12 (both pro-

inflammatory) and IL-10 (anti-inflammatory) (7, 106). More specifically, *in vitro* studies show, for instance, that both TLR2 and TLR4 mycobacterial triggering are important for the production of TNF (108-111) and that TLR2 and TLR9 are important receptors for driving IL-12 production in response to mycobacteria (108, 109, 112). Also, mycobacterial TLR2 engagement has been shown to be crucial for IL-10 production by antigen presenting cells (APCs) (113, 114).

Despite the fact mice deficient for myeloid differentiation primary response gene (88) (MyD88), an adaptor molecule for most of the TLRs, have decreased protection against *M. tuberculosis* infection (115-117), the *in vivo* relevance of TLRs remains controversial as several studies show different results. These differences, however, might be related to different experimental set ups. In response to a low dose (50-200 colony forming units (CFU)) of *M. tuberculosis* aerosol infection, the protection level achieved by mice deficient for TLR2 (109, 118), TLR4 (109, 119, 120) or TLR9 (108) is similar to the one presented by wild type mice. However, in response to a high dose (500-2000 CFU) of *M. tuberculosis,* both TLR2 (109, 118) and TLR9 (108) deficient mice have higher bacterial burdens and reduced survival time when compared to wild type counterparts. Regarding the response of TLR4 deficient mice, there are conflicting results where these mice show either no differences in protection in respect to wild type mice (109, 120) or lose the ability to control infection (121, 122). Moreover, TLR2 and TLR9 were shown to be necessary for the formation of a proper granuloma structure (108, 118, 123). Additionally, TLR9 triggering defects were shown to result also in reduced IL-12 production (123). When deficiency is both for TLR2 and -9, even when a low dose of *M. tuberculosis* is used, mice become susceptible to infection showing lower levels of pro-inflammatory cytokines as well as decreased survival (108). Conversely, mice deficient for TLR2, -4 and -9 at the same time, and infected with a low dose of inoculum, despite presenting an impaired pro-inflammatory cytokine production, show similar levels of protection as compared to wild type mice (117).

In what refers to the association of human TLR2, -9 and -4 with tuberculosis, a link remains to be established. Despite some studies associate some polymorphisms in *TLR2* or TLR2 signaling molecules with susceptibility to tuberculosis (124-129), in some populations this association has not been established (130, 131). As well, *TLR4* polymorphisms have been shown to associate with susceptibility to tuberculosis in some populations (132, 133) but not in others (134, 135). Finally, *TLR9* polymorphisms have also been shown to associate with disease (128),

however the role of TLRs on the host immune response is still not totally elucidated, as different human studies and different mice models of infections reveal different results (7).

More studies are needed to further elucidate the specific contribution of TLRs for the outcome of the host/pathogen interplay.

1.4.1.2 Toll like receptors and signaling pathways.

All TLRs share the same protein motif in their cytoplasmatic region: Toll/IL-1R (TIR) domain (136). In response to different stimulus, this TIR domain allows for the binding between TLRs and TIR-domain containing adaptor molecules which can be of four types: MyD88, TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1 (TICAM-1), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL) and TRIF-related adaptor molecule (TRAM) (95, 137). MyD88 is used by all except TLR3, which uses uniquely TRIF. TLR4 is the only TLR that uses both MyD88 and TRIF adaptors, although at different stages of TLR4 signaling (95). Moreover, the MyD88 adaptor molecule has a crucial role in the protection against *M. tuberculosis* infection since in its absence mice infected with *M. tuberculosis* show lower levels of pro-inflammatory cytokines, uncontrolled bacterial burden, and animal death (115-117).

Once MyD88 is recruited, signaling pathways such as the nuclear factor (NF)-kB transcription factor (138) and mitogen-activated protein (MAP) kinases (MAPKs) (95, 139, 140) are activated thereby leading to the expression of cytokines and chemokines (98, 100, 141). The nuclear factor (NF)-kB pathway is activated by any TLR (98, 104) and leads to the expression of several important cytokines, such as IL-10 and TNF(95, 142, 143), representing a very important pathway for the host immune response to pathogens as it is the case of mycobacteria (144-147). In mice deficient for (NF)-kB, infection by mycobacteria leads to multifocal necrotic pulmonary lesions and a general decrease in the expression of pro-inflammatory molecules (148). In regard to the MAPKs, there are at least three subgroups of MAPKs (140, 149): Jun amino-terminal kinases (JNK) (150, 151), p38 (152-154) and extracellular signal-related kinases (ERK)-1/2 (154, 155), all identified as important signaling molecules activated by mycobacteria (156). In monocytes from active pulmonary tuberculosis patients, the activation of both p38 and ERK leads to an increased IL-6 and TNF production as compared to monocytes from healthy donors (157). Interestingly, the activation of p38 and ERK decreases overtime, as well as pro-inflammatory cytokine production, in response to pathogenic mycobacteria, when compared to non-pathogenic strains (158). In response to mycobacterial challenges, p38 and ERK phosphorylation leads to

production of TNF (159-161) and IL-10 (in the case of p38 phosphorylation) (114, 162). Additionally, p38 is also associated with the activation and apoptosis of peripheral mononuclear cells from tuberculosis patients (163) and with the arrest of *M. tuberculosis* phagosome maturation (164).

Although there are some data showing the involvement of MAPK in cytokine production in response to mycobacteria, more studies are needed to further define how these signaling pathways regulate the immune response, namely at the level of cytokine gene expression/regulation.

1.4.1.3 Pattern Recognition Receptors other than toll like receptors

In regard to PRRs, other than TLRs, involved in the recognition of mycobacterial PAMPs, NOD2, DC-SIGN and dectin-1 have been identified as taking part of the pathogen recognition and with roles in the host immune response (76, 165).

NOD2 is an intracellular PRR and presents leucin-rich repeats similar to those found in TLRs and recognizes peptidoglycans from both Gram-positive and Gram-negative bacteria (166). NOD2 has been shown to be involved in mycobacterial recognition (94, 167, 168) and its absence results in impaired cytokine production (168, 169). However, studies using NOD2 deficient mice infected with *M. tuberculosis* show contradictory results regarding protection since these mice have bacterial burdens that are either similar or higher than the ones in wild type mice (169, 170).

Dectin-1 is a transmembranar C-type lectin receptor and it typically recognizes β-1, 3 glucans (fungi PAMPs) (171). Heat stable compounds, possibly lipoglycans and α–glucans, from *M. tuberculosis* cell wall have been proposed to be recognized by dectin-1 (76, 172, 173) but the precise mycobacterial ligands that trigger this receptor needs further investigation. Dectin-1 triggering activates an adaptor molecule called spleen tyrosine kinase/Caspase recruitment domain-containing protein 9 (CARD9) and, as consequence, NF-kB becomes activated and cytokine production begins (171, 174). The engagement of Dectin-1 in the context of mycobacterial infection is important for IL-12p40 production by DCs (172) and results in the induction of adaptive cell responses (175), however, upon infection dectin-1 deficient mice have bacterial burdens similar to the ones from wild type mice (174, 176). Conversely, when CARD9 is absent, mice present impaired cytokine production, high bacterial burdens and rapidly die from

infection (177), however, this adaptor molecule is activated by several PRRs so the susceptibility to infection might be related to other receptors besides Dectin-1.

Finally, DC-SIGN, also a C-type lectin receptor, has been identified as a target for certain mycobacterial PAMPs, such as Man-LAM (178, 179). Despite of being involved in mycobacterial recognition (179) phagocytosis (89, 90), DC-SIGN triggering also dampens DCs maturation by limiting the expression of co-stimulatory molecules (178). Upon infected with a high dose of *M. tuberculosis*, mice deficient for the homologous receptor of DC-SIGN show increased bacterial burdens as compared to wild type mice (180). Furthermore, a transgenic mouse model expressing human DC-SIGN under the CD11c promoter indicates that this receptor might be helpful to limit pathology and to increase survival (181).

Overall, studies regarding the innate immune recognition of mycobacteria show that these pathogens are recognized by many PRRs among which TLRs play fundamental roles and cooperate with other receptors. Further attention needs to be dedicated to this subject to shed light in the role of PRRs during infection.

1.4.2 The innate immune response

The innate immune response represents the first line of defense against mycobacteria, namely in the lung, and, although crucial to initiate the fight against the pathogen (72), this type of immunity by itself is not enough to contain the infection (9, 73, 77). This arm of immunity comprises mainly macrophages, DCs, natural killer cells (NK), neutrophils, monocytes, eosinophils, and γδ T cells. Although innate cells protect the host in a non specific manner and do not confer long lasting protection, unlike adaptive cells, they are essential to generate and modulate microbicidal mechanisms adaptive immune responses. Some of these cells not only recognize and respond to mycobacteria but are also able to phagocytose them. *In vivo* and *ex vivo* studies in mice have shown that upon aerosol infection both *M. tuberculosis* and *M. bovis* BCG are able to infect various cells such as alveolar macrophages or lung DCs (78-82, 182). Also neutrophils (83-85), eosinophils (183) and epithelial cells (86, 87) have been identified as mycobacteria-harboring cells. Soon after mycobacterial infection, macrophages stay at the site of infection initiating microbicidal mechanisms, while DCs migrate to the draining lymph nodes (DLNs) where they will drive T cell activation and differentiation (73, 90). In addition, neutrophils and NK cells migrate to the inflammatory sites and contribute for the host immune response,

either by killing directly mycobacteria and mycobacteria bearing cells or by producing cytokines that help macrophages to fight infection.

1.4.2.1 Macrophages and microbicidal mechanisms

Macrophages sense the mycobacteria, phagocyte it and several microbicidal mechanisms are initiated such as phagosome/lysosome fusion (184-187), generation of reactive nitrogen and oxygen intermediates (RNI and ROI) (77, 188, 189) and apoptosis (190-192).

Upon fusion of the phagosome with the lysosome (184, 185), the inside of this new structure becomes highly acidic and oxidized and, as a consequence, hydrolytic enzymes are activated plus toxic oxidative compounds are generated, leading to mycobacterial killing (188, 189). However, *M. tuberculosis* has developed strategies to escape the phagosome-lysosome fusion (193-195), which greatly accounts for mycobacterial survival and intracellular growth (193).

Regarding the respiratory burst, the inducible isoform of nitric oxide synthase 2 (iNOS2) activity on L-arginine, generates nitric oxide and its metabolites (RNI) leading to very potent mycobacterial killing (189, 196, 197). In fact, mice deficient on iNOS2 are highly susceptible to mycobacterial infections (198-200). Moreover, it has been shown that inhibition of iNOS in human alveolar macrophages infected with BCG leads to increased mycobacterial growth (201, 202). Concerning ROI, it results from the transfer of an electron from NADPH to molecular oxygen by NADPH-oxidase and although it is considered to be involved in mycobacterial killing, this process might not be exceptionally important since mice lacking NADPH-oxidase are only mildly/not susceptible to infection (200, 203).

Importantly, IFN-γ and TNF are key players at guaranteeing that the above described antimicrobial mechanisms are fully accomplished (9, 77). A great deal of the TNF production comes from the macrophage itself and acts autocrinally but it is also produced by T helper cells, whereas the majority of IFN-γ comes initially from NK cells and later from CD4+ Th cells (77). Upon macrophage activation via TNF and IFN-γ, iNOS2 is induced (204) leading to the production of RNI (199). The lack of IFN-γ or TNF signaling in mice also associates increased susceptibility to *M. tuberculosis* (205-208) and *M. bovis* BCG (209). Moreover, TNF deficiency also results in defective granuloma formation, due to compromised cell recruitment (210). In humans the anti-TNF therapies conducted in patients with rheumatoid arthritis are associated with an increased incidence of tuberculosis, most probably due to reactivation of latent disease

(211). As well, humans that lack IFN-γ signaling seem to be at greater risk to develop tuberculosis (52, 212, 213).

Finally, apoptosis of infected macrophages, as a microbicidal mechanism, limits the pathogen growth (214, 215) and in humans, this process has been shown to be dependent on TNF signaling. Also, increasing mycobacterial virulence seems to be associated with decreased apoptosis (214). In fact, an increased mycobacterial virulence is associated with lower TNF levels due to the negative regulation promoted by higher IL-10 levels which, in turn, correlated with increased mycobacterial virulence (216).

All these reports collectively show how important macrophages (and monocytes) are in the immune response to mycobacteria thus putting together a series of microbicidal mechanisms at the site of infection, namely the lung.

1.4.2.2 Dendritic cells

DCs are crucial for B and T cell activation (217) and together with macrophages, produce different types of cytokines able to regulate the migration, expansion and differentiation of B and T cells (75, 99, 217). The migration of infected DCs to the DLN is a milestone for the immune response against mycobacteria since it allows the adaptive immune response to be initiated (73). Once mycobacteria is sensed and phagocytosed, immature DCs acquire a mature and activated phonotype which is characterized by the up - regulation of MHCII and MHCI, adhesion molecules and co-stimulatory molecules such as CD40, C80 and CD86 (218-220). Interestingly, depending on the infecting mycobacteria and experimental setting, DCs will mature differently. For instance, DCs infected with *M. bovis* BCG, are able to become efficient APCs (78, 221), in contrast to *M. tuberculosis* infected DCs that are less efficient at activating Ag85 transgenic CD4+ Th cells (78, 221).

Once matured, DCs will increase their cytokine production (218, 220, 222) and migrate to the DLNs (78, 80, 223, 224). The p40 homo-dimer, IL-12$(p40)_2$, IL-12p70 (composed by the subunits p40 and p35) and IL-23 (composed by the subunits p19 and p40) are essential pro-inflammatory cytokines produced by DCs in response to mycobacterial challenges (225). IL-12$(p40)_2$-producing DCs are able to migrate to the DLNs in response to subcutaneous antigen delivery (226) and, in response to *M. tuberculosis*, IL-12$(p40)_2$ deficient DCs increase IL-10 expression and fail to migrate to the DLNs (227). Moreover, mycobacteria-infected DCs that produce IL-12p70 will lead to the generation of protective IFN-γ producing Th1 cells. In fact,

mutations in the IL12p40 and IL-12 receptor genes in humans are associated with lower IFN-γ production by Th1 cells and with increased susceptibility to disseminated *M. bovis* BCG and *Mycobacterium avium* infections (228-230). In the animal model, CD4+Th cells transferred into Rag deficient mice are protective but not when the recipients are concurrently IL-12p40/ Rag deficient (231) and, in fact, mice that lack IL-12p40 are highly susceptibility to *M. tuberculosis* infection failing to generate protective IFN-γ responses (232, 233). Also, the protection afforded by *M. bovis* BCG against *M. tuberculosis* infection is improved when IL-12 was used as an adjuvant during immunization, resulting in a lower bacterial burden as compared to mice that received BCG alone for immunization (234). Moreover, IL-12p70 although expressed at low levels in the lung might be required at higher levels in the draining lymph modes where the generation of IFN-γ producing Th1 cells takes place (233). Actually, IL-12p70 has been shown not only to be the key element in driving IFN-γ Th1 mycobacterial specific responses (227) but also to be required for the maintenance of their effector function in the lung (231). Interestingly, in mice that lack IL-12p35 and consequently IL-12p70, IL-23 seems to replace this cytokine by inducing IFN-γ responses (235), however, this replacement is only temporary and mice ultimately succumb to infection (233).

Conversely to IL-12 deficiency, in the absence of IL-23p19, mice are still protected against primary mycobacterial infections (235, 236), showing bacterial burdens, numbers of Th1 cells and short-term survival similar to wild-type mice (235). The Th17 population, however, as well as the IL-17 mRNA levels, is impaired when IL-23 signaling is absent (235, 236). The exact way of how Th17 cells are derived by DCs signals in response to mycobacterial challenges is still poorly understood. However, some reports have suggested that TGF-β plays a fundamental role in the generation of these cells in response to mycobacteria or other stimuli (237, 238) but only if IL-6 is present (237), otherwise, when the cytokine balance is towards TGF-β it will drive the differentiation of a T naïve into a regulatory T cell (Treg) response (239). In addition, IL-1 and TNF have been suggested to have an additive contribution for the Th17 differentiation, but these cytokines, either alone or in combination, by themselves, are not sufficient to support the Th17 cell differentiation (240). Regarding the role of IL-23 on Th17 cells, it has been suggested that this cytokine acts not as an inducer of this cell type but rather as a survival factor (235, 241). In fact, DCs activated with mycobacterial stimuli are able to drive the generation of Th17 which will only be able to persist if IL-23 is present (235, 237, 241-243). Furthermore, although IL-23 signaling does not seem to interfere with a protective immune response to primary mycobacterial

infections (235, 236), it seems to improve protective efficacy of vaccination when used as an adjuvant (243) and to be crucial at inducing a Th17 memory population that is protective in a vaccination context against tuberculosis (242).

Altogether, the above mentioned studies uncover DCs as fundamental players in the host immune response to mycobacterial challenges and further and more detailed studies on how DCs respond to mycobacteria are of major interest.

1.4.2.3 Other cells from the innate arm of immunity

Neutrophils are, due to their phagocytic capacity and microbicidal mechanisms (*eg.* oxidation, granule proteins and iron-withholding molecules) (244, 245), efficient cells at fighting infection. Despite of the microbicidal mechanisms of neutrophils and of their recruitment to the sites of mycobacterial infection (246), studies where mice were depleted for granulocytes show contradictory results regarding the contribution of these cells for protection against mycobacteria either reporting no influence in protection or associating them with protection (247, 248). Recently, *Berry M et al* (249) identified a IFN-inducible neutrophil-driven human blood transcriptional signature that correlate with the clinical severity of human tuberculosis, hence, due to their cytotoxic nature, a tight control of the neutrophil response is necessary otherwise leading to pathological tissue damage in response to infection (61, 246).

NK cells are involved in protective immunity against several pathogenic agents, including mycobacteria (250). NK cells are the early key IFN-γ producers soon after intracellular pathogen infection (251) contributing for the initiation of the microbicidal mechanisms on macrophages. NK cells are also able to kill infected cells as shown, as shown in studies that report human NK cells killing *M. tuberculosis* infected phagocytes (252-254), namely human alveolar macrophages (252). However, although NK cells accumulate in the lungs of mice and produce IFN-γ in the first 2 weeks post *M. tuberculosis* infection, mice depleted of these cells do not become more susceptible (255). This suggests that NK cells, although able to mount an adequate immune response to *M. tuberculosis*, might have functions that are overlapped by other immune cells (102).

γδ T cells are a subtype of T cells, but classically belong to the innate immune type of response. In the context of mycobacterial infections these cells rapidly respond and contribute for pathogen clearance through the production of granulysins that directly kill either extracellular or intracellular mycobacteria (256). Moreover, γδ T cells have also been reported to be the major

source of IL-17 production in response to mycobacterial infections (257-259). In the murine model, the absence of these cells results in defective granuloma formation, however, the bacterial loads are reported to be either similar to that of wild type mice (260) or higher in the early phase of infection (261). Therefore, the precise role of these cells in mycobacterial infections remains unclear (262).

Collectively, the innate immune system gathers several cell types able to initiate important microbicidal mechanisms and also contribute for the initiation and modulation of adaptive immune responses that are essential for the outcome of the host immune response to mycobacteria.

1.4.3 The adaptive immune response

The adaptive immune response is essential for the control of infection with intracellular pathogens such as *M. tuberculosis* and consists in the activation and differentiation of T cells which are able to amplify the microbicidal mechanisms of macrophages and lyse infected cells (73, 77).

The protective host immune response against mycobacterial challenges is intrinsically connected to CD4+Th cells as, in resistant mice, the transition from the mycobacterial exponential growth to the bacteriostatic stage takes place upon the arrival of CD4+Th cells to the infected organ (263). Moreover, in response to mycobacteria, CD4+Th deficient mice have increased bacterial burdens and reduced survival rates when compared to wild type mice (264, 265), presenting also an impaired granuloma formation (265). In humans, the relevance of these cells is reiterated on HIV infected patients who possess a severe deficiency on the CD4+Th cell compartment rendering these patients more prone to develop tuberculosis (73, 266, 267).

The generation of T cell protective responses takes place in the DLNs but only upon arrival of bacteria with antigen availability in this location (224, 268). Furthermore, by taking advantage of T cell receptor (TCR) transgenic mice specific for mycobacterial antigens, several studies showed that T cell activation in the DLNs takes place around 8 to 10 days upon challenge (224, 268, 269). Once naïve T cells are differentiated into effector cells, they migrate towards the lung (224, 268) arriving 14 days post infection when bacteria is within alveolar macrophages and DCs (78). Moreover, IFN-γ, the hallmark cytokine of Th1 cells, greatly potentiates the macrophage mycobactericidal mechanisms (204, 270, 271) and in its absence mice become susceptible to infection with either non-virulent or virulent mycobacteria (205, 207, 272, 273). In

humans, deficiencies in IFN-γ or IFN-γ receptor have been shown to be associated with increased susceptibility to both *M. tuberculosis* and *M. bovis* BCG (-osis) (52, 230).

In addition to CD4+Th1 cells, Th17 cells are also present during the immune response to mycobacteria (235, 241, 274) and their response is greatly impaired in the absence of IL-23 (235, 275). Moreover, IFN-γ, by inhibiting the IL-23 production by APCs, negatively regulates Th17 cells (241). In the absence of IL-17, protection is not altered in response to a low dose of *M. tuberculosis* infection (275), however, if the infection dose is increased both granuloma formation and protection become impaired (258). Nonetheless, Th17 cells gain more value in the context of vaccination, where they seem to be able to anticipate the recruitment of Th1 cells to the lung thus promoting increased resistance to mycobacterial infection (242). However, as a result of repeated *M. bovis* BCG vaccination, IL-17 responses are increased and uncontrolled thus inducing tissue damage associated with an increase in neutrophil recruitment to the lung (61). Therefore, Th17 cells need to be tightly regulated during mycobacterial infections otherwise resulting in serious tissue damage.

Th2 cells differentiate in response to IL-4 and are classic key players in the protective immunity to extracellular parasites since they activate humoral responses with production of IgG1 and IgE (276, 277). Depending on the genetic background of mice, the lack of a typically Th2 cytokine, IL-4, either does not alter the mycobacterial bacterial burdens, when compared to wild type mice (278), or results in increased protection (279). As for Th2 role in human tuberculosis, IL-4 responses seem in some cases to associate with disease (280), however the role of these cells in protection against tuberculosis is still obscure essentially mainly because mycobacteria, such as *M. tuberculosis*, are intracellular organisms thereby mainly activating Th1, or even Th17, types of response.

In response to mycobacteria CD8+T cells produce IFN-γ (281) and have the ability to produce granzymes consequently leading to death of infected macrophages (282). Despite their microbicidal capacity, and that MHCI deficient mice become more susceptible to mycobacterial infection than wild type mice (264, 283), CD8+ T cell deficiency does not promote the drastic failure in protection against mycobacteria that is observed for MHCII or CD4+ T cell deficiencies (9).

Foxp3+ regulatory T (Treg) cells have been shown to be increased in patients with active tuberculosis, when compared to latent individuals, and to inhibit protective Th1 responses, allowing for mycobacterial growth and tissue damage (284). Furthermore, mice infected with

mycobacteria show that Tregs proliferate and accumulate at the infection sites (285) and that these cells inhibit protective responses important for the control of *M. tuberculosis* (285), thus preventing mycobacteria eradication by dampening otherwise efficient CD4+T cells (286). In this study, suppression by Tregs was not via IL-10 and in the absence of these cells protection was not related to increased IFN-γ amount, TNF or nitric oxide (NO) production (286).

B cells, apart of being professional APCs and producing antibodies, have also the ability of stimulate the differentiation of Th1 cells and modulate cytokine response of macrophages and DCs (287). Although antibodies are not able to enter the cytoplasm of mycobacteria-infected cells, when the pathogen is exposed, as it is the case before being phagocytosed or when host cells are lysed, antibodies are then able to help mycobacterial elimination (287). In a murine model where no mature B cells are present, so no antibody production takes place, mice become more susceptible to *M. tuberculosis* and *M. bovis* BCG infections when compared to wild type mice (288, 289) and show exacerbated immune pathology associated with elevated pulmonary recruitment of neutrophils (289). Furthermore, human antibodies, generated in response to *M. bovis* BCG vaccination, have the ability to promote protective responses against *M. bovis* BCG infection and also lead to the proliferation of IFN-γ CD4+Th cells and CD8+ T cells (290). However, there are also studies using B cell deficient mice that, upon mycobacterial infection, behave in a similar way as wild type counterparts (291, 292). Therefore, the role of B cells in protective immunity against mycobacteria still needs further clarification.

1.5 IL-10: an important modulator of the immune response

As mentioned previously, in the context of mycobacterial infections the production of inflammatory cytokines is of major importance for a protective host immune response. Nevertheless, balance between pro-inflammatory, such as IFN-γ, and anti-inflammatory cytokines, such as IL-10, is important to dictate the course of infection as well as tissue damage.

IL-10 is an anti-inflammatory cytokine produced and recognized by several cells from both the innate and adaptive arms of immunity (293). Accordingly, among innate cells, macrophages and DCs (but not plasmocytoid DCs, as referred by Boonstra *et al* (294)) are the main producers of IL-10, although mast cells, eosinophils, monocytes, neutrophils, and NK cells are also able to produce this cytokine (293). Within the adaptive arm of immunity, B cells, Th2, Treg cells, Th1 cells, Th17 cells and CD8+T cells have been also shown to produce IL-10 (293).

IL-10, by binding to its receptor, a dimeric transmembranar protein (295), engages the Jak family tyrosine kinases Jak1 and Tyk2, which become phosphorylated (296, 297) leading to the activation of transcription factors, such as STAT3, that are responsible for the suppressive action of IL-10 (295, 298).

The anti-inflammatory action of IL-10 has a broad range of targets acting in an autocrine/paracrine way, mainly on the innate arm of immunity, namely on macrophages and DCs. In this regard, IL-10 is capable of: i) inhibit the production of pro-inflammatory cytokines such as IL-1, IL-6, TNF and IL-12 (294, 299-301), *eg.* in the context of LPS or *Staphylococcus* enterotoxin B superantigen stimulation (301, 302); ii) diminish the expression of chemokines like CCL2 and 5, IL-8, CXLCL2 and 10 (295); iii) down-regulate the expression co-stimulatory proteins on macrophages (303) and on monocytes (*eg.* CD80 and CD86) (304); and iv) down-regulate MHCII molecules thereby diminishing antigen presentation and dampening T cell activation (295, 299). In what regards the adaptive arm of immunity, IL-10 directly compromises T cell differentiation, by dampening IL-12 production by APCs (295), and function by impairing cytokine production, namely of IFN-γ, IL-4 IL-5 and IL-2 (305). Conversely, IL-10 presents positive effects on certain cells, which is the case of B cells, by promoting their proliferation and immunoglobulin production (306). On CD8+T cells, IL-10 stimulates their cytotoxic activity (307), recruitment (308) and proliferation (309). In addition, IL-10 acts as a differentiation and survival factor for induced regulatory T cells (310).

1.5.1 IL-10 in infections

In response to invading pathogens the host immune system engages both innate and adaptive immune responses in an attempt to eliminate the threat. However, during the process of immune activation, deregulated antimicrobial responses can promote tissue damage to the extent that they may become more hurtful than the infection itself (295). Thus, ideally, the host immune response should be regulated in such way that, although able to respond robustly and long enough to eliminate microbes, tissue damage is prevented. Therefore, suppressive signals such as TGF-β and IL-10 need to be in place to control and mediate excessive host immune reactions thereby contributing for the balance between pathogen clearance and tissue damage (295, 311). Interestingly, depending on the type of infection, the presence of IL-10 may either contribute for host protection or susceptibility. In certain cases IL-10 presence is key to avoid immune-pathology, as shown by IL-10 deficient mice that develop colitis (312) in response to gut

flora (313, 314) and severe immune pathology during infections by *Toxoplasma gondii* (315-317), *Plasmodim chabaudi* (318-320) and *Helicobacter hepaticus* (321). Furthermore, it has been shown that administration of recombinant IL-10 or IL-10 over-expression in transgenic mice leads to reduced levels of TNF, IFN-γ and MIP-2 thereby enhancing survival during toxic-shock-syndromes (322, 323). Conversely, IL-10 can also lead to impaired immune responses, or even to chronic infections. In fact, increased pathogen clearance is observed during infections with *Leishmania major* (324), *Listeria monocytogenes* (325) and *Candida albicans* (326) when IL-10 is decreased. Moreover, extreme or ill-timed IL-10 production have deleterious effects for hosts infected with *Tripanossoma cruzi* (327), *Plasmodium yoelli* (328), and *Leishmania spp* (329, 330) or presenting lymphocytic chroriomeningitis (331) to the point where the pro-inflammatory responses are impaired leading to either rapid or chronic non-healing disease. Altogether, these studies indicate that IL-10 activity result in different outcomes.

1.5.2 IL-10 in mycobacterial infections

The role of IL-10 in human mycobacterial infections has been vastly studied (332). However, the precise role of this cytokine in the immune response remains to be elucidated. There are a number of studies suggesting diverse suppressive effects of IL-10 upon mycobacterial challenges, on different immune cells and their products, such as cytokines. Accordingly, *in vitro* studies show an increase in T cell proliferation from active tuberculosis patients (333) and an increase in IFN-γ production when IL-10 is neutralized (333, 334). Similarly, IL-10 was found to inhibit proliferation and IFN-γ production by human CD4+Th and γδ T cells in response to *M. tuberculosis* challenge (335). The suppressive effects of IL-10 on the adaptive immunity to mycobacteria are many times indirect since IL-10 acts mostly on innate immunity, *eg.* APCs, by: i) inhibiting the production of pro-inflammatory cytokines such as TNF and IL-12 in macrophages and DCs (218, 222, 334, 336) which in turn compromises the production of IFN-γ (334, 335, 337) and lytic activity of T cells (337); ii) blocking phagocytosis and mycobacteria killing by reducing the production of ROI and RNI in response to IFN-γ (295, 338); iii) inhibiting IL-12p40 dependent migration of DCs from the mycobacteria infected lung towards the draining lymph nodes (227, 336); iv) down-regulating co-stimulatory proteins like CD40, CD80 and CD86 (334, 335, 337) and the chemokine IP-10 (339); v) blocking apoptosis of human alveolar macrophages infected with *M. tuberculosis* (216) or murine APCs (340).

Some human data suggests the genetic association between *IL-10* polymorphisms and susceptibility to tuberculosis. One example is a human case control study in Gambia in which variations in the allele 2 of *SLC11A1* (*Nramp1*) were found to be associated with increased IL-10 production by monocytes, as well as other innate cells, and with increased susceptibility to primary progressive tuberculosis (341). Also, depending on the geographic location and ethnicity of the studied population, some human *IL-10* polymorphisms were shown to be associated with susceptibility to tuberculosis (342-346). More recently, a meta-analysis revealed no association between the three studied *IL-10* polymorphisms and tuberculosis risk, however, a subgroup analysis by ethnicity revealed that Europeans with a particular IL-10 genotype have a decreased risk of tuberculosis (347). Further studies with increased number of subjects are necessary to bring additional information on the importance of IL-10 for tuberculosis susceptibility.

Even though the genetic association studies have not found a clear association between IL-10 and susceptibility to tuberculosis, the fact is that this cytokine is increased during human active pulmonary tuberculosis in the lung (10, 348, 349), serum (350) and bronchoalveolar lavage (BAL) fluid (mainly comprised by macrophages and neutrophils) (349). Also, in the sputum of active patients enhanced levels of IL-10 were found which positively correlated with the increased levels of the CFP32 antigen from *M. tuberculosis*, which could imply an association between IL-10 and a failure to contain infection (10).

As for the source of IL-10 during *M. tuberculosis* infection, Gerosa *et al* (351) found that CD4+T cells isolated from broncheoalveolar lavage (BAL) of active tuberculosis patients, produce IFN-γ as well as IL-10 (351). Interestingly, genetically modified mice to over-express IL-10 by the T cell compartment have enhanced susceptibility to *M. bovis* BCG due to impaired co-stimulatory macrophage functions (352). These mice are also more susceptible to *M. tuberculosis* due to impaired Th1 responses, as the number of activated Th1 cells was diminished in the blood and lung tissue (353). Another cellular source of IL-10 during *M. tuberculosis* infection are alveolar macrophages and lung DCs that upon aerosol infection become rapidly exposed to the pathogen, phagocytose it and produce not only TNF and IL-12 but also IL-10 (218, 219, 222, 354). In fact, in transgenic mouse models where macrophages over-express IL-10, although cytokine production remains unaffected, there is a strong impairment in RNI production, an increase in arginase-1 and an overall diminished macrophage microbicidal function (355). These results relate nicely to another study showing that one of the molecules that regulate arginase-1 is IL-10

(356). Moreover, it has been shown that over-expression of IL-10 by macrophages also leads to the inhibition of TNF in response to *M. avium* (357).

Although the results obtained from studies with transgenic mice that over-express IL-10 are difficult to compare side by side, since they differ, for instance, in the mycobacterial species used, the readouts, as well as in the cellular source of IL-10, the reality is that irrespective of all those conditions, whenever IL-10 is over-expressed the outcome reflects a marked inability to mount a protective immune response against mycobacteria. It is thus reasonable to consider that depending on the stage and anatomical location of the infection, the cellular source and the amount of IL-10 produced, the modulation of the immune response will vary.

There is vast literature agreeing on the IL-10 detrimental effects on the immune response to mycobacteria, however, some contradictory results arise when we try to associate absence of IL-10 with resistance/susceptibility to *M. tuberculosis* infection. In view of that, infected IL-10 deficient mice in C57BL/6 background, although having enhanced levels of IFN-γ (358), show to be either only transiently protected (359) or to have similar bacterial burdens as wild type mice (358, 360), which is in contrast with a recent finding from Redford *et al* (339) showing that IL-10 deficient mice become more susceptible at chronic stages of infection. Furthermore, at very late stages of infection these mice succumb and show increased bacterial burdens, tissue damage and increased pro-inflammatory cytokine production (361). When IL-10 deficient mice, in Balb/c background, are challenged with *M. tuberculosis* there is also protection associated with lower bacterial burdens and increased pro-inflammatory cytokine production (339). In line with this, when IL-10 is neutralized from Balb/c mice, *M. avium* clearance is enhanced (362, 363). Furthermore, when IL-10 is depleted during the chronic stage of infection, CBA/J mice, that by default is susceptible to *M. tuberculosis*, show an increased survival accompanied by diminished bacterial burdens and increased IFN-γ production (364).

Interestingly, even for the same mouse strain the role of IL-10 has different outcomes depending on the mycobacterial infecting species; IL-10 deficient C57BL/6 mice that have a sustained increase of IFN-γ producing T cell responses associated with increased clearance of *M. avium*, meanwhile, in response to *M. tuberculosis*, the increase of IFN-γ T cell responses and bacterial burdens are only transient (359). In addition to this, regardless of the mouse strain, the lack of IL-10 seems to facilitate the production of cytokines and chemokines important for the granuloma formation (339, 365).

These studies, by showing different infection outcomes associated with differences on the mouse strain, are in line with findings of the above mentioned human studies which show that depending on the genetic background of the host, namely due to different ethnicity, alterations in the production of IL-10 may or may not associate with susceptibility to *M. tuberculosis*. Importantly, IL-10 seems to have the potential of modulating the efficacy of *M. bovis* BCG vaccination as it is shown by *Gopal R et al* (366), which suggests that in the absence of IL-10 the efficacy of *M. bovis* BCG is improved in an IL-17–independent manner.

Altogether, whether the IL-10 suppressive effects reflect a positive balance to the host by avoiding excessive inflammation accompanied by tissue damage, or if it is one of the reasons why chronicity is achieved, is a matter yet to be discovered and that is most probably related with the type of infection, immune background of the host, anatomical location/cellular source of IL-10 and level of IL-10 being produced at specific stages of the infection. Thus, further insights on how IL-10 regulation occurs in innate cells is a matter that still needs additional investigation and which could be helpful to identify possible manipulation targets in favor to the host.

1.5.3 IL-10 regulation, expression and production by innate cells

In vivo, the ability of macrophages and DCs to produce IL-10 in response to PRRs triggering has been shown (293). In the specific case of TLR engagement, IL-10 induction requires the activation of the signaling adaptor molecules MyD88 and TRIF and of a panoply of indirect pathways that are coordinated by autocrine and paracrine factors (293, 294, 300, 367-369). In fact, LPS–stimulated macrophages show full IL-10 production only when both MyD88 and TRIF are activated (294, 368) and type I interferon signaling is in place (368). TLR triggering leads to the activation of MAPK signaling cascades and NF-kB (370) and, in the case of IL-10 induction, the MAPKs ERK and p38 are key players. Accordingly, upon TLR2, 4 or 9 stimulation both ERK and p38 become activated leading to IL-10 production (300, 371-374), as well as other cytokines such as TNF (161, 375). Moreover, increasing levels of ERK activation result in increased amounts of IL-10 production (371) and in inhibition of IL-12 production in macrophages and DCs (300, 371, 374). In the absence of ERK signaling, either by chemical inhibition (300, 371, 374) or in ERK deficient cells (376), TLR-induced IL-10 production is greatly impaired in DCs. Upstream of ERK is the complex NF-kB/TPL2 that upon TLR stimulation becomes dissociated and TPL2 is then able to activate ERK (377, 378). However, in the absence of NF-kB, TPL2 is rapidly degraded and ERK activation becomes compromised (379).

Furthermore, upon TLR stimulation, TPL2 deficient APCs are not able to phosphorylate ERK thereby producing lower amounts of IL-10 as compared to wild type cells (371). Also, in response to TLR stimulation, chemical inhibition of NF-kB (143) or macrophages deficient for NF-kB (372) result in diminished IL-10 production. Moreover, in the absence of the complete NF-kB pathway, macrophages stimulated with LPS have their levels of TNF and IL-10 impaired (especially IL-10) (380).

Regarding the involvement of p38 on IL-10 induction it has been shown that in its absence, IL-10 expression is compromised in TLR stimulated macrophages (373, 374, 381), DCs (382) and peripheral blood monocytes (383). Also, in the absence of the p38 regulator dual specific protein phosphatise 1 (DUSP1), macrophages have prolonged p38 phosphorylation and increased IL-10 production (384-386). This IL-10 increase is dependent on the p38 activation since it can be reversed chemically inhibiting this MAPK (384). Importantly, addition of exogenous IFN-γ to macrophages results in diminished p38 and ERK phosphorylation and impaired IL-10 promoter activation accompanied by decreased IL-10 production (387). Furthermore, IL-10 is able to negatively regulate itself by inducing DUSP1, which blocks p38 activation, thus impairing IL-10 expression (388). Conversely, on primary macrophages, IL-10 can also promote a positive loop of self-regulation since it can induce TPL2 expression (389).

The precise molecular mechanisms that lead to cytokine gene expression are of major importance. In this sense an important layer of regulation for the expression of several cytokines is the modulation of mRNA stability (390). Many cytokine genes present a 3´untranslated region (UTR), containing class II adenosine-uridine-rich elements (AREs) which represent locations that expose mRNAs to degradation. This is the case of IL-10 mRNA that contains several AREs in its 3´UTR (391). Interestingly, TLR-stimulated blood monocytes can produce IL-10 which induces its own mRNA degradation (392). However, as compared to other mRNA cytokines that are rapidly degraded in response to IL-10, this cytokine acts only later on to induce its own mRNA destabilization, via an unknown IL-10-induced molecule (392). A molecule shown to bind the AREs of IL-10 mRNA, thereby inducing mRNA degradation, is a zinc finger protein called tristetraprolin (TTP) (393). In fact, LPS stimulated macrophages deficient for TTP have lower IL-10 mRNA decay and consequent higher IL-10 production (393). Additionally, p38 induces the phosphorylation, and consequent deactivation, of TTP resulting in lower IL-10 (and TNF) mRNA decay rates (394-396). Moreover, microRNAs (miRs) have been also described as having a role in the regulation of IL-10 (397), as it is the case of the microRNA miR–106a (398) and the miR–

98 (399) that bind to IL-10 3´UTR thereby inducing IL-10 mRNA degradation and decreased production. Conversely, miR–4661 and miR–21 positively regulate IL-10 production by antagonizing TTP mediated IL-10 mRNA decay (400) and by blocking PDCD4, a suppressor of IL-10 transcription (401).

The ability to sense the pathogen, to initiate microbicidal mechanisms and to modulate adaptive immune responses is of major importance to generate protective immune responses. Upon mycobacterial recognition, cytokine production is a major step for the initiation of these events, therefore, to study how the expression of cytokines is regulated is of major importance. In the particular case of IL-10, despite the conflicting human data linking IL-10 with susceptibility to tuberculosis, mice that either over express or are deficient for IL-10 reveal the importance of this cytokine at different stages of infection mediating both innate and adaptive cell responses. Macrophages and DCs are among the first cells that sense mycobacteria, therefore, the elucidation of the mechanisms that regulate IL-10 expression in these cells upon infection is of major interest and are still unclear. The role of TLRs in protection against human tuberculosis remains also to be further clarified and, in the specific case of TLR2, it seems to depend greatly on the ethnic background of the studied population. Detailed studies describing how TLR2 recognition impacts the generation of protective immune responses, namely adaptive immune responses, are still missing and might be of major help to further understand host/pathogen interactions and to identify new targets for the design of new vaccines.

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CHAPTER 2

OBJECTIVES

Tuberculosis, caused by *M. tuberculosis*, is a serious threat of public health. Protective immune responses are associated with various pro-inflammatory cytokines, however, avoidance of pathology has to be achieved by a fine tuned balance involving both pro- and anti-inflammatory cytokines, such as IL-10. Therefore, is of utmost importance to understand the regulation of IL-10 upon the early recognition of the pathogen by innate cells. Furthermore, the association between early recognition and possible impact at the level of Th responses, is of great interest. The specific contribution of TLR2 on Th17 type of responses remains also elusive. Therefore, the specific aims of the studies, which lead to the results presented in this dissertation, were:

(i) TO CHARACTERIZE THE MACROPHAGE IL-10 RESPONSE UPON INFECTION WITH DIFFERENT STRAINS OF *M. tuberculosis*.

Considering the relevance of macrophages on the innate response to mycobacteria and the detrimental role of IL-10 on immune responses, the first aim of the present study was to follow the dynamics of expression and production of IL-10 upon infection with mycobacteria known to activate preferentially TLR2 or TLR4. This analysis was intended to characterize the mechanisms operating to regulate IL-10, namely at the post-transcriptional level. For this objective, we took advantage of the *in vitro* generation of wild type, TLR2-/-, TLR4-/- and TRIF-/- macrophages.

(ii) TO DISSECT THE CYTOKINE PRODUCTION BY DENDRITIC CELL BY COMPARING INFECTION WITH *M. tuberculosis* AND *M. Bovis* BCG.

The protection against tuberculosis afforded by *M. bovis* BCG has been described as variable and of short duration. Therefore, we aimed to dissect the IL-10 and IL-12 responses induced by *M. bovis* BCG in what compares to those driven by *M. tuberculosis*. For this objective we derived wild type, TLR2-/- and TLR9-/- DCs *in vitro*.

(iii) TO INVESTIGATE THE IMPACT OF TLR2 TRIGGERING ON THE DEVELOPMENT AND ESTABLISHMENT OF TH17 CELLS UPON *M. tuberculosis* INFECTION.

Th17 cells have been shown to have an important role during recall responses to *M. tuberculosis* infections for the recruitment of protective Th1 cells. Some studies have shown the involvement of Th17 cells in the context of mycobacterial infections, however, both the role of

these cells and the involvement of TLR recognition on this type of responses in the context of mycobacterial infections have been poorly studied. Taking this into consideration, we aimed to investigate the biological relevance of TLR2 recognition in the generation and maintenance of Th17 cells upon infection with *M. tuberculosis*. For this we used the *in vivo* wild type and TLR2-/- mouse models and infected them intranasally with *M. tuberculosis*.

CHAPTER 3

EXPERIMENTAL RESULTS

CHAPTER 3.1

TLR2 and TLR4 signals differently regulate IL-10 expression by macrophages at the post-transcriptional level

TLR2 and TLR4 signals differently regulate IL-10 expression by macrophages at the post-transcriptional level. Maria Teixeira-Coelho[1,2], Joana Guedes[1,2], Jenny Carmona[1,2], Pedro Ferreirinha[1,2], Ashleigh Howes[3], John Ewbank[3], Lúcia Moreira-Teixeira[1,2], Jorge Pedrosa[1,2], Anne O´Garra[3], António G. Castro[1,2], and Margarida Saraiva[1,2]. *In preparation*

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ABSTRACT

Interleukin (IL)-10 is widely expressed by cells of the immune system. The molecular mechanisms that regulate IL-10 expression are tightly controlled to ensure an effective immune response in the absence of pathology. Stimulation of macrophages by toll-like receptors (TLRs) leads to IL-10 expression, with the known involvement of MAP kinases (MAPKs). However, how macrophages regulate their IL-10 production upon recognizing live microorganisms via TLRs remains less studied. To answer this question, we compared the regulation of IL-10 production by macrophages infected with two strains of *Mycobacterium tuberculosis*. We found that, depending on the specific activation of TLR2 or TLR4, the IL-10 mRNA had different stability profiles. TLR2 signals promoted a rapid induction and degradation of IL-10 mRNA, whereas those of TLR4 led to IL-10 mRNAs with increased stability. By using single TLR2 and TLR4 agonists, we found that TLR4 signaling induced prolonged p38 activation, via TRIF, which was responsible for the increased stability of the IL-10 mRNA. We thus reveal a novel pathway of IL-10 regulation, at the post-transcriptional level, that links TLR triggering, the TRIF pathway and p38 activation. Our study suggests that differential recognition of pathogens by TLRs has an impact on the amount of IL-10 produced by macrophages.

INTRODUCTION

Interleukin (IL)-10 is a powerful anti-inflammatory cytokine produced by many cells of the immune system, namely innate immune cells such as macrophages (1). The interplay between IL-10 and a pro-inflammatory response during infection is essential to achieve a balance where the clearance of infection does not correlate with immune pathology (2). *Mycobacterium tuberculosis* is the causative agent of Tuberculosis (TB), which represents a problem of public health that still nowadays contributes to high rates of morbidity and mortally (3). In the context of *M. tuberculosis* infection, IL-10 has the ability to impair both the adaptive immune response (4-9), that is essential for the control of infection (10, 11), and the innate immune response (5, 7, 8, 12-14). Studies using mouse models that lack IL-10 present disperse results regarding the outcome of infection by *M. tuberculosis* (15, 16), probably due to the usage of different mouse and mycobacterial strains. However, in mouse transgenic models where IL-10 is over expressed, either by the T cell or the macrophage compartment, a marked inability to control mycobacterial infection was reported (5, 17, 18). Indeed in the context of IL-10 over expression mice succumbed earlier to infection (18) even when the T cell IFN-γ responses were intact (17, 18). Moreover, human studies show that IL-10 is increased in the lungs (19-22) and serum of active pulmonary TB patients (23). Furthermore, variations in *SLC11A1* (Nramp1), a TB susceptibility locus, are linked to enhanced production of IL-10 and such variations are associated with increased susceptibility of individuals to pulmonary TB (24).

Considering the dampening effects of IL-10 on protective immunity, a tight regulation of this cytokine production needs to take place in any cell that is able to produce it. IL-10 has been described to be regulated at different levels such as via chromatin remodeling, triggering of mitogen activated protein kinases (MAPKs) and post-transcriptional mechanisms (1). These layers of IL-10 regulation have been studied in several IL-10-producing cells, including in toll-like receptor (TLR)-activated macrophages (1). TLR activation initiate a series of signaling pathways by recruiting adaptor molecules such as myeloid differentiation primary response protein 88 (MyD88), in the case of TLR2 and TLR4, and TIR-domain containing adaptor protein inducing IFN-β (TRIF), in the case of TLR4, thereby leading to the activation of innate cells like macrophages (25, 26). As a result of the signaling events triggered by TLR activation through single ligands or complex pathogens (1, 16), various cytokines are produced, including IL-10 (27, 28). The MAPKs p38 (29-31) and ERK (27, 29, 32, 33) have been reported to be important for the induction of IL-10 production in the context of TLR stimulation and p38 has been further

implicated in the post-transcriptional regulation of IL-10 by helping to protect the IL-10 mRNA from rapid degradation induced by tristetraprolin (TTP), an RNA binding protein (34).

In this study, we analyzed the molecular mechanisms regulating IL-10 production by macrophages in response to two different strains of *M. tuberculosis*, *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen. Previous data from our laboratory indicate that these strains are differently recognized by TLR2 and TLR4 (Carmona J *et al*, submitted). We found that the differential recognition of the two *M. tuberculosis* strains by TLR2 or TLR4 impacted the amount of IL-10 produced by macrophages. More specifically, TLR2 activation led to rapid degradation of IL-10 mRNA whereas TLR4 activation promoted IL-10 RNA stability. This increased IL-10 mRNA stability was due to TRIF-dependent TLR4 activation that allowed for prolonged p38 phosphorylation. Therefore, our study unveils a role for TRIF, through p38 activation, in the post-transcriptional regulation of IL-10 in macrophages.

MATERIAL AND METHODS

Animals. All animals used were females with eight to twelve weeks on the C57BL/6 background. Wild type (WT) mice were ordered from Charles River (Barcelona, Spain). TLR2-/- and TLR4-/- animals were maintained at ICVS and TRIF-/- were from MRC-NIMR. All mouse protocols followed the European Union Directive 86/609/EEC and were previously approved by the national authority *Direcção Geral de Veterinária*.

Bacteria. *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen strains were grown in Proskauer Beck (PB) medium containing 0.05% Tween 80 to mid-log phase and frozen in PB medium and 30% glycerol at –80°C. The DH5X strain of *Escherichia coli* was grown in Lysogeny broth (LB) medium to mid-log phase and frozen in PBS 1X and 30% glycerol. *E. coli* was heat inactivated prior to cell stimulation during 1hr at 65°C. All bacteria were added to the cell cultures at a multiplicity of infection of two.

Cell culture. For cell culture cDMEM (complete Dulbeco´s modified Eagle´s medium - GIBCO) was prepared by supplementing DMEM with 10% FCS, 1% sodium pyruvate, 1% hepes and 1% L-glutamine (GIBCO). Briefly, to derive macrophages, $4x10^6$ bone marrow cells were plated in a petri dish (STERLIN) using 8 mL of cDMEM supplemented with 20% of L929-cell conditioned media (LCCM) and by day 4 the cells were fed with 10 mL of the same media. Macrophages were derived during 7 days of culture under 37°C and 5% CO_2 conditions, after which cells were collected, counted and stimulated.

Cell culture Reagents. All reagents were prepared as indicated by each company: LPS, Actinomycin D (ActD) (Sigma) and Pam3Cys (InvivoGene) were used at 25ng/mL, 10μg/mL and 2μg/mL, respectively. p38 (SB0203580) and ERK (PD0325901) inhibitors were used at 2,5μM and 0,1μM, respectively. MAPK inhibitors and ActD were added to the cell cultures 50 min and 1hr, respectively, post stimulation either with mycobacteria or single ligands. DMSO (cell culture grade) was obtained from Sigma.

ELISA. Cell culture supernatants were collected at the indicated time points and kept at - 80 °C until an ELISA was performed to specifically measure IL-10, following the manufacturer's instructions (eBioscience).

RNA extraction, cDNA and quantitative real time PCR (RT-PCR). Total RNA from stimulated and non-stimulated cell culture samples was extracted with TRIzol® 143 Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA was synthesized using a reverse transcriptase reaction according to the manufacturer (Fermentas) and on the MyCycler™ thermal cycler (Bio-Rad). Relative gene expression of IL-10 was assessed using the SYBR Green Supermix as recommended by the company (Fermentas) and performed using the CFX96™Real-Time System (Bio-Rad). Ubiquitin was the chosen reference gene. The sequences for the ubiquitin and IL-10 were designed and synthesized by TIB MolBiol: ubiquitin forward, GCTGGTGAAAAGGACCTCT; ubiquitin reverse, CACAGGACTAGAACACCTGC; IL-10 forward 5'- TTT GAA TTC CCT GGG TGA GAA -3'; and IL-10 reverse 5'- GCT CCA CTG CCT TGC TCT TAT T -3'.

Western Blot (WB). Cells were derived as mentioned above and by day 7 were replated and rested for 5 hours in 1% FCS – cDMEM prior to stimulation. At the indicated time points the stimulation media was discarded and wells gently washed with apyrogenic PBS 1X (GIBCO). Protein extracts were obtained with a lyses buffer solution (100mM Tris-HCl pH8; 10% glycerol; 1mM EDTA pH8; 5mM MgCl2; 50 mM NaCl; 1% NP-40; 1x protease inhibitor cocktail from Roche; 1x phosphatase inhibitor cocktails II and III from Sigma; dH2O) for 20 minutes on ice. Extracts were kept at -80°C until further use. The lysates were then centrifuged at 13000 rpm for 15 minutes and protein extracts recovered rejecting the pellet. Immediately before use, protein extracts were heated 5 minutes at 95°C and 20µg of each sample resolved in a 12% SDS-PAGE and then transferred by using the nitrocellulose membranes Trans-Blot Turbo Transfer Pack (0,2µM) from Bio-rad in the machine Tran Blot Turbo-Transfer system from the same company. Membranes were incubated with antibodies against total p38, phospo p38, total ERK and phospho ERK (all from Cell Signaling) following the respective manufacturer's instructions and used at 1:1000. The secondary antibody was anti-rabbit (Cell Signaling) and used at 1:10000. The membranes were developed with SuperSignal Femnto reagent (Thermo Scientific) and read by a Universal Hood II (Bio-Rad). Quantity one (Bio-Rad) was the software used to analyze the results.

Statistical analysis. The data are expressed as Mean ± SEM and was analyzed using the two-tailed Student´s t test. The p values considered as having statistical significance were $0,05 \leq p^*$, $0,01 \leq p^{**}$ and $0,001 \leq p^{***}$.

RESULTS

Distinct IL-10 mRNA degradation rates are promoted in macrophages upon recognition of genetically related strains of *M. tuberculosis.* The role of IL-10 in response to mycobacterial challenges has been a matter of intensive study in the past years (16). We have previously shown that two genetically related *M. tuberculosis* strains, *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen (both belonging to the Euro – American lineage), induce different amounts of IL-10 secretion by macrophages (Carmona J. *et al*, submitted and Fig. 1A). To further elucidate the molecular mechanisms underlying these differences, we started by analyzing the kinetics of IL-10 transcription induced in macrophages upon stimulation with each bacteria. Although at 1hr post stimulation both *M. tuberculosis* strains induced a peak of IL-10 mRNA (Fig.1B), in response to *M. tuberculosis* H37Rv that peak was lower and also less sustained throughout time than what was observed in response to *M. tuberculosis* Harlingen. Considering the apparent differences observed in terms of the maintenance of the IL-10 mRNA peak with either *M. tuberculosis* strains between 1h and 3hrs post stimulation, we questioned if these two strains were inducing distinct IL-10 mRNA degradation rates in macrophages. To test this hypothesis, we evaluated the stability of the IL-10 mRNA 1hr post stimulation and throughout the next 90 min using ActD, a drug that blocks transcription. In agreement with our hypothesis, Fig. 1C shows that the stability of the IL-10 mRNA induced by *M. tuberculosis* Harlingen is higher than that observed for *M. tuberculosis* H37Rv. Collectively, our results showed that genetically related strains of *M. tuberculosis* lead to different amounts of IL-10 production by infected macrophages, being the regulation of the IL-10 mRNA stability one of the underlying mechanisms.

Figure 1.

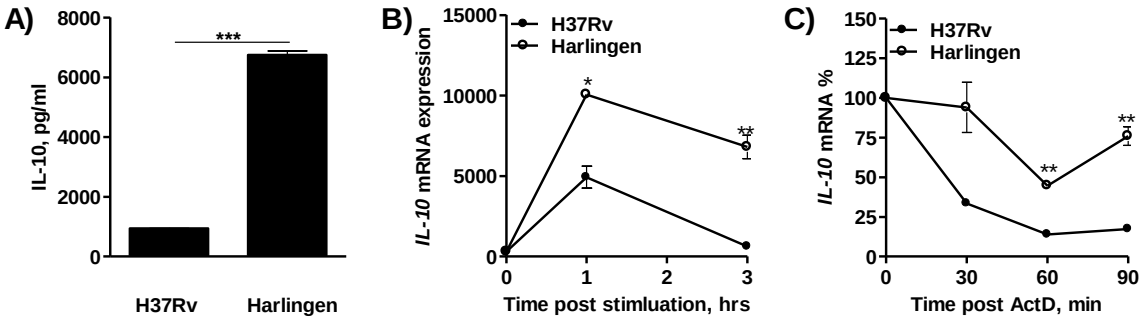

Figure 1. Distinct IL-10 mRNA degradation rates are promoted in macrophages upon recognition of genetically related strains of *M. tuberculosis.* WT macrophages were stimulated with *M. tuberculosis* H37Rv or *M. tuberculosis* Harlingen at a MOI of 2 (A-C). 6hrs post stimulation cell culture supernatants were collected and the amount of IL-10 measured by ELISA (A). When indicated, ActD was added to cell cultures 1 hr post stimulation. At the indicated time points post stimulation (B) and post ActD addition to cell culture (C) RNA was extracted, converted to cDNA and the expression of *IL-10* was analyzed by RT-PCR and normalized to the expression of ubiquitin. Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the Student´s t test (*p<0.05; ***p<0.001). The data are representative of three independent experiments.

***M. tuberculosis* triggering of TLR4, but not TLR2, protect IL-10 mRNA from rapid degradation.** TLRs, namely TLR2 and TLR4, have been implicated in the recognition of mycobacteria (35-37) and their triggering is capable of inducting IL-10 (1). We have previously shown that *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen are differentially recognized by TLR2 and TLR4 (Carmona J *et al*, submitted). Therefore, we next asked if the different ability of *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen at inducing IL-10 production was, at least in part, dependent on distinct recognition of mycobacteria by TLR2 and TLR4. For that, we started by comparing the amount of IL-10 produced by macrophages in the absence of TLR2 or TLR4 upon stimulation with *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen. As shown in Fig. 2A, in response to *M. tuberculosis* H37Rv the IL-10 production by macrophages was severely impaired in the absence of TLR2, but it was not altered in the absence of TLR4. Conversely, in response to *M. tuberculosis* Harlingen stimulation, the production of IL-10 by macrophages was only modestly dependent upon TLR2 signals, but greatly dependent on TLR4 recognition (Fig. 2A). Similar findings were observed at the transcriptional level (Figs. 2B and C). These findings led us to ask if differential TLR2 and TLR4 signaling was promoting different IL-10 mRNA

degradation rates, being TLR4 a protective signal for IL-10 mRNA degradation. To test this hypothesis, we stimulated TLR2-/- or TLR4-/- macrophages with *M. tuberculosis* Harlingen and, at 1hr post stimulation, measured the stability of the IL-10 mRNA by using ActD. As shown in Fig. 2D, as compared to WT macrophages, although TLR2 absence promoted some decrease of IL-10 mRNA stability, in the absence of TLR4 the IL-10 mRNA stability was highly compromised, being comparable to that observed upon infection of WT macrophages with *M. tuberculosis* H37Rv (Fig. 1C). Interestingly, upon exposure of macrophages to *M. tuberculosis* Harlingen, the lack of TLR4 not only significantly compromised the overall IL-10 mRNA induction but also did not allowed for a sustained IL-10 mRNA expression between 1hr and 3hrs (Fig. 2C).

In all, our results show that the differential recognition of *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen strains by TLR2 and TLR4 impacted the stability of the IL-10 mRNA.

Figure 2.

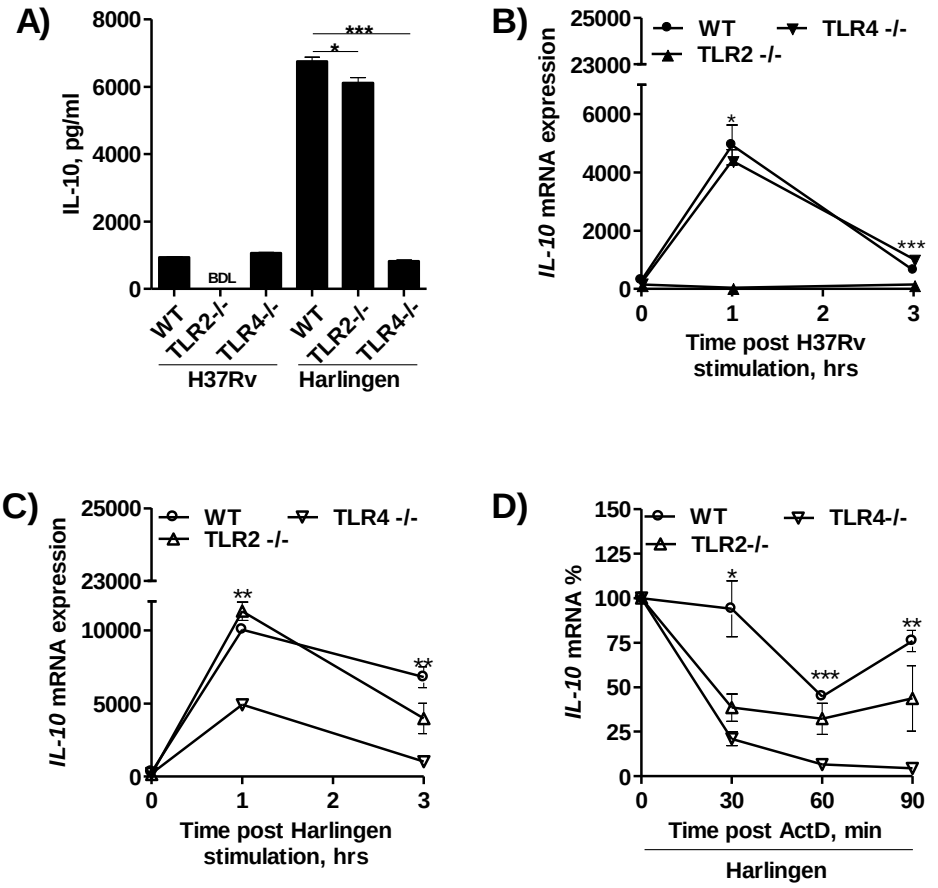

Figure 2. *M. tuberculosis* triggering of TLR4, but not TLR2, protect IL-10 mRNA from rapid degradation. WT, TLR2-/- and TLR4-/- macrophages were stimulated with *M. tuberculosis* H37Rv or *M.*

tuberculosis Harlingen (A-D). Cell culture supernatants were collected 6 hrs post stimulation and the amount of IL-10 was measured by ELISA (A). When indicated, ActD was added to cell cultures 1 hr post stimulation. At the indicated time points, post stimulation (B, C) and post ActD addition to cell culture (D), RNA was extracted, converted to cDNA and the expression of *IL-10* was analyzed by RT-PCR and normalized to the expression of ubiquitin. Results are representative of two independent experiments and are Mean ± SEM value for 3 replicates per group. The p values were determined by the Student´s t test (*p<0.05; ***p<0.001). Statistics refer to comparison between WT and TLR2-/- (B) and between WT and TLR4-/- in (C and D).

TLR4 signals induce IL-10 mRNA stability in macrophages. Considering that mycobacteria are complex organisms that activate a variety of PRRs on innate cells (35-37) we next asked if the effects promoted by TLR2 and TLR4 on IL-10 mRNA regulation would be recapitulated by using specific single ligands for these two receptors. For that, we simulated macrophages with LPS and Pam3Csk, TLR4 and TLR2 ligands, respectively. Similarly to what was observed for *M. tuberculosis* Harlingen *versus M. tuberculosis* H37Rv strains, LPS promoted a lower degradation of the IL-10 mRNA, as compared to Pam3Csk (Fig. 3A). In line with this, although the IL-10 mRNA levels detected at 1hr post-stimulation were similar to both ligands, over time there was a sustained IL-10 mRNA amount in response to LPS, but not to Pam3Csk (Fig. 3B). In line with these differences, the IL-10 production by macrophages was higher in response to LPS than to Pam3Csk (Fig.3C). In addition, we also found that the IL-10 gene transcribed by macrophages in response to *Escherichia coli* (a mainlyTLR 4 – activating bacteria, Supp. Fig. 1A) is stable over time (Supp. Fig. 1B). Altogether, these results indicate that IL-10 expression is differently regulated by TLR2 and TLR4 at the post-transcriptional level.

Figure 3

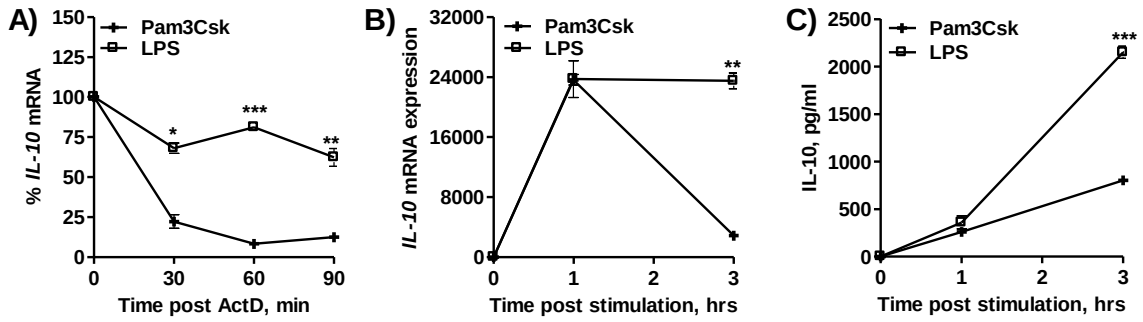

Figure 3. TLR4 signals induce IL-10 mRNA stability in macrophages. WT macrophages were simulated with Pam3Csk or LPS (A-C). When indicated, ActD was added to cell cultures 1 hr post stimulation. At the indicated time points post ActD (A) and post stimulation (B), RNA was extracted, converted to cDNA and the expression of *IL-10* was analyzed by RT-PCR. Cell culture supernatants were collected at the indicated time points post stimulation and the amount of IL-10 measured by ELISA (C). Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the Student´s t test (*p<0.05; ***p<0.001). The data are representative of two independent experiments.

Prolonged p38 phosphorylation is required for TLR4 - mediated IL-10 mRNA stability. Both p38 and ERK phosphorylation are required for IL-10 induction by innate immune cells in the context of TLR stimulation (1). Additionally, it has been reported that p38 activation is linked to molecular mechanisms controlling IL-10 mRNA stability (1, 34). We thus hypothesized that differential IL-10 mRNA stability observed upon TLR2 *versus* TLR4 triggering could be related to differential p38 activation. To evaluate this, macrophages were stimulated with either strain of *M. tuberculosis*, LPS or Pam3Csk and a Western Blot was performed to assess the phosphorylation levels of p38 and ERK overtime. Despite differences in the intensity of the activation of p38 and ERK upon TLR2 or TLR4 triggering, a higher and prolonged activation of p38 induced by TLR4 signaling was particularly noticeable (Fig. 4A-E). We next investigated if the inhibition of p38 activation would impact the stability of the IL-10 mRNA observed upon TLR4 triggering. To study this, macrophages were stimulated either with *M. tuberculosis* Harlingen or with LPS and a chemical p38 inhibitor was added to the cell cultures. The p38 inhibitor was added 50 minutes post-stimulation, so that the initial transcription of the IL-10 gene was not affected. In response to *M. tuberculosis* Harlingen (Fig. 4F) and to LPS (Fig. 4G), the inhibition of p38 phosphorylation resulted in decreased stability of IL-10 mRNA. Furthermore, lower amounts

of IL-10 secreted by stimulated macrophages in the presence of p38 inhibitor were observed (Fig. 4H). Of note, we have used two other p38 inhibitors and the same results were obtained (data not shown). Furthermore, inhibition of p38 also led to IL-10 mRNA degradation (Supp. Fig. 1C) and to lower IL-10 secretion (Supp. Fig. 1D) by macrophages stimulated with *E. coli*.

In what concerns a role for ERK in the post-transcriptional regulation of IL-10, we found that chemically inhibition of ERK during TLR4 activation did not lead to alterations in the IL-10 mRNA degradation rates (Supp. Fig. 2A and 2B). However, in agreement with previous studies (1), inhibition of ERK phosphorylation compromised IL-10 production by TLR-stimulated macrophages (Supp. Fig. 2C).

In sum, our data indicate that TLR4 triggering in macrophages leads to higher and more sustained p38 phosphorylation than TLR2 triggering, which dictates different post-transcriptional mechanisms in place to regulate IL-10 expression by these cells.

Figure 4

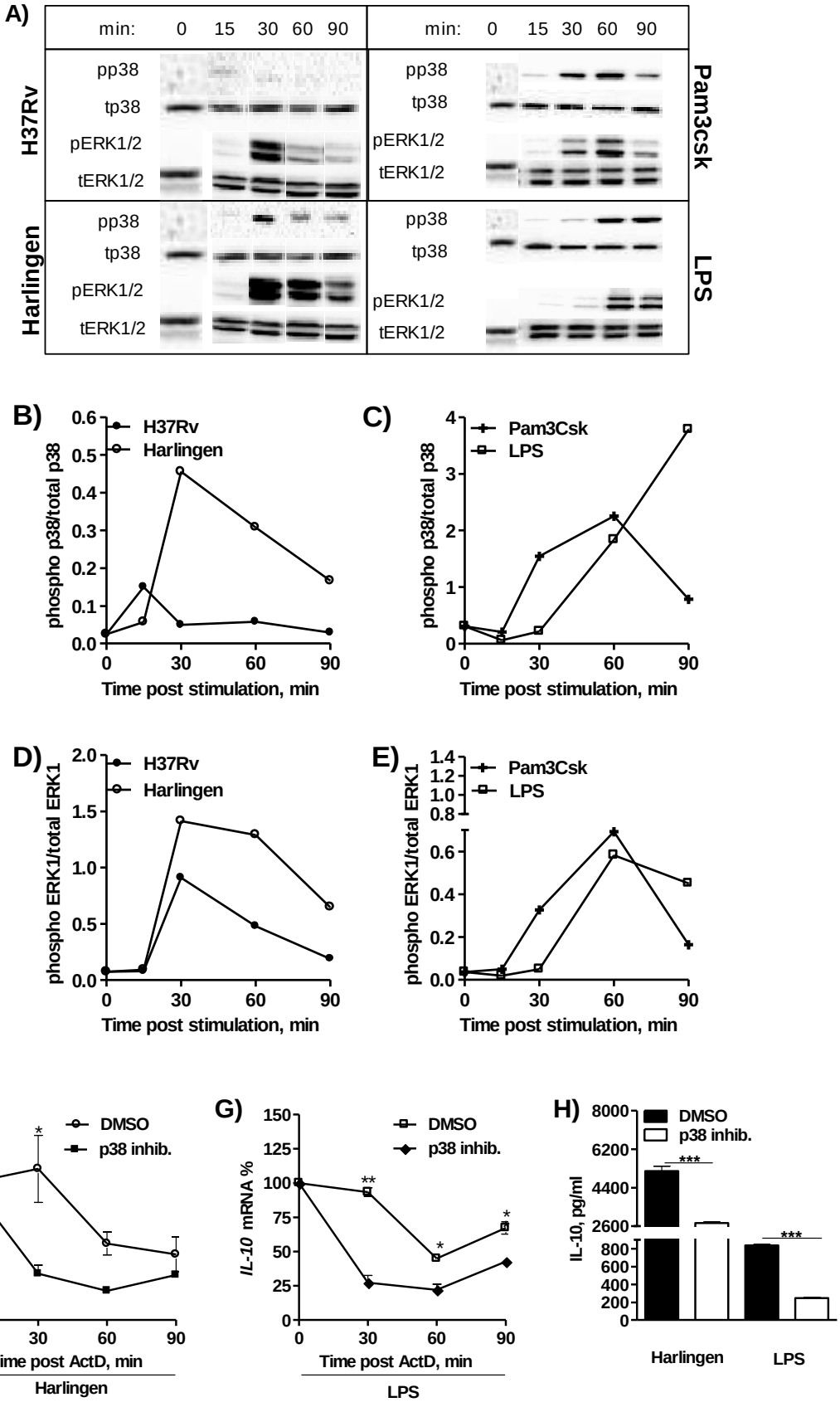

Figure 4. Prolonged p38 phosphorylation is required for TLR4-mediated IL-10 mRNA stability. WT macrophages were simulated with *M. tuberculosis* H37Rv, *M. tuberculosis* Harlingen,

Pam3Csk or LPS (A-H). At the indicated time points post stimulation protein extracts were obtained and WB for p38 and ERK performed (A). WB quantification of phospho p38/total p38 and phospho ERK/total ERK ratios, results are 3 pooled replicates per group (B-E). When indicated, p38 inhibitor was added 50 min post stimulation and ActD added 1hr post stimulation. At the indicated time points post ActD addition to cell culture, RNA was extracted, converted to cDNA and the expression of *IL-10* was analyzed by RT-PCR (F, G). Cell culture supernatants were collected 6hrs post stimulation and the amount of IL-10 measured by ELISA (H). Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the Student´s t test (*$p<0.05$; ***$p<0.001$). The data are representative of three independent experiments.

TRIF signals sustain the p38 activation needed for IL-10 mRNA stability. The signaling pathways triggered by TLR2 and TLR4 have in common the activation of the cascades mediated by the adaptor molecule MyD88 (38). However, TLR4 further signals through the adaptor molecule TRIF (26). We next questioned whether TLR4-derived TRIF signaling was the pathway implicated in the sustained p38 phosphorylation and required for the stability of the IL-10 mRNA. To test this, WT and TRIF-/- macrophages were generated and stimulated with *M. tuberculosis* Harlingen or with LPS. In the absence of TRIF signaling, both prolonged p38 phosphorylation (Fig. 5 A - C) and IL-10 mRNA stability (Fig. 5 D - E) were impaired and the amounts of secreted IL-10 decreased (Fig. 5F). Collectively, these results uncover a crucial role for TLR4 induced TRIF activation at protecting IL-10 mRNA from rapid degradation though induction of strong and sustained p38 phosphorylation.

Figure 5

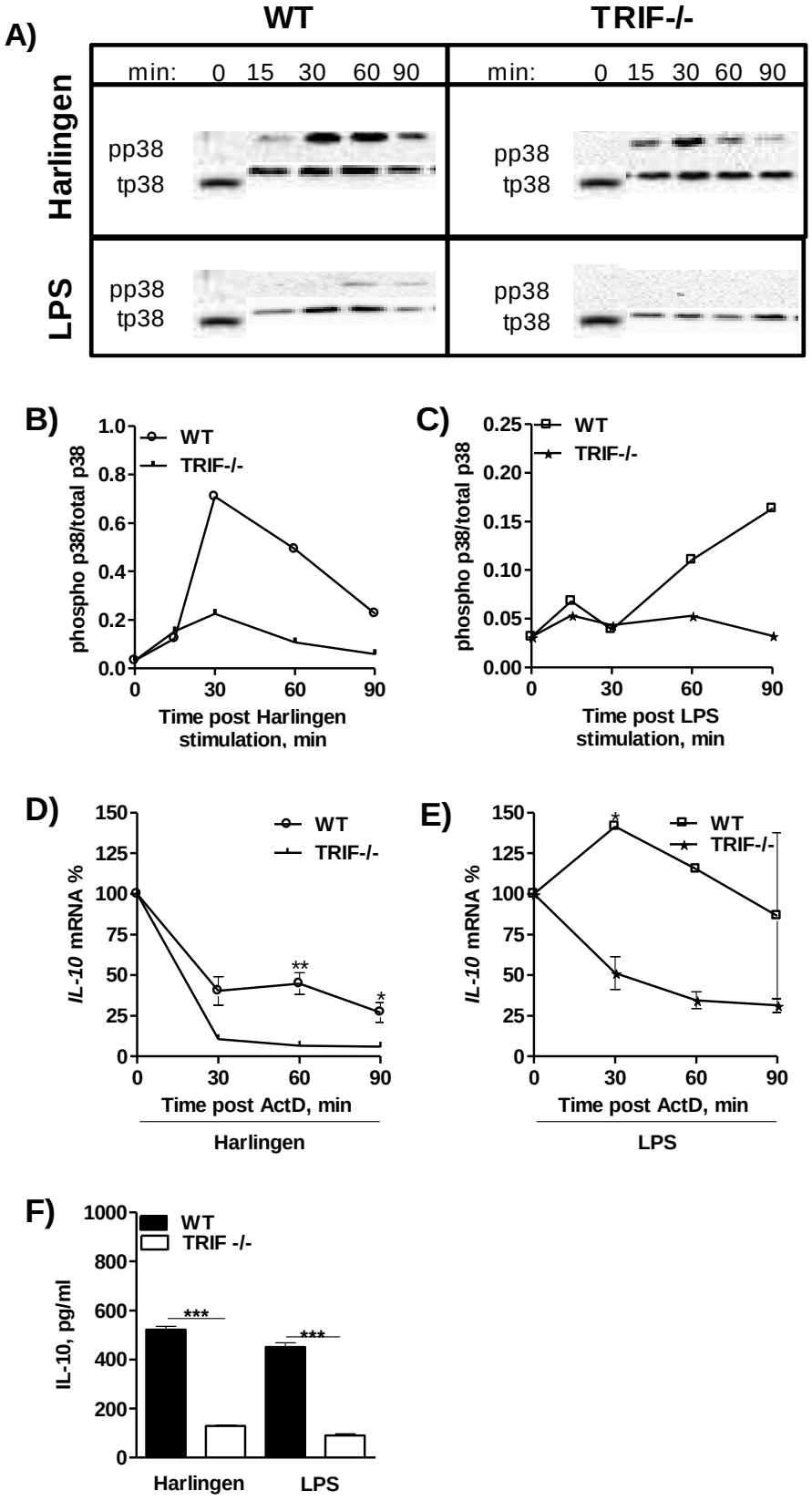

Figure 5. TRIF signals sustain the p38 activation needed for IL-10 mRNA stability. WT and TRIF-/- macrophages were simulated with *M. tuberculosis* Harlingen or LPS (A-F). At the indicated time

points post stimulation protein extracts were obtained for WB and phosphor p38 or total p38 levels assessed (A). WB quantification of phospho p38/total p38 ratios in response to *M. tuberculosis* Harlingen and LPS, respectively; results are 3 pooled replicates per group (B, C). When indicated, ActD was added to cell cultures 1 hr post stimulation. At the indicated time points post ActD addition to cell culture RNA was extracted and the expression of IL-10 was analyzed by RT-PCR (D, E). Cell culture supernatants were collected 6hrs post stimulation and the amount of IL-10 measured by an ELISA assay (F). Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the Student´s t test (*$p<0.05$; ***$p<0.001$) (D-F). The data are representative of one experiment.

DISCUSSION

IL-10, due its vast anti-inflammatory properties (39), has the ability of shaping the immune response to infectious agents, with implications in the balance between pathogen clearance and immune pathology (16, 39). Therefore, the study of the molecular mechanisms regulating IL-10 production by immune cells is of importance. Several mechanisms have been implicated in the regulation of IL-10 expression, from specific signaling cascades and transcription factors, to chromatin remodeling or to post-transcriptional mechanisms (1). To date, although several reports indicate that IL-10 translation is a highly regulated process (34, 40-45), the fact is that post-transcriptional mechanisms of IL-10 gene regulation have been largely neglected. In the present work, we show that the IL-10 mRNA degradation is differently regulated by TLR2 and TLR4 signals in macrophages. More specifically, we found that TLR4-induced TRIF activation promoted higher and prolonged phosphorylation of the MAPK p38 than TLR2 mediated signals. This sustained p38 activation was the key event to protect IL-10 from mRNA rapid degradation and this only occurred upon TLR4 activation of macrophages. As consequence of the different IL-10 mRNA degradation, macrophages ultimately secreted variable amounts of IL-10 in response to TLR2 or TLR4 stimuli.

Interestingly, this post-transcriptional mechanism for IL-10 regulation operated in macrophages upon differential recognition of two *M. tuberculosis* strains by TLR2 and TLR4. Indeed, we found that *M. tuberculosis* Harlingen, that preferentially activates TLR4, induced higher IL-10 production by these cells than *M. tuberculosis* H37Rv, a strain of *M. tuberculosis* preferentially recognized by TLR2. The ability of mycobacterial challenges to differently modulate mRNA stability of molecules other than IL-10 has been shown before (46-49). To date, the mechanisms underlying IL-10 mRNA degradation have been studied in the context of single ligand stimulation, mainly LPS (34, 50, 51). Now, with this work, we are able to begin to understand how IL-10 is differently regulated in response to complex organisms such as mycobacteria. Interestingly, among the panoply of receptors and signaling pathways that mycobacteria trigger and activate on macrophages (52), we found that two important receptors for IL-10 regulation are TLR2 and TLR4. We observed that *M. tuberculosis* strains that activate different TLRs, induced different amounts of IL-10 secreted by macrophages and investigated the molecular mechanisms underlying the observed differences. The ability of TLR4 to protect IL-10 from rapid mRNA degradation as compared to TLR2 was further confirmed by stimulating

macrophages with LPS and Pam3Csk which recapitulated the profile from *M. tuberculosis* Harlingen and *M. tuberculosis* H37Rv stimulation, respectively.

Upon TLR engagement the phosphorylation of MAPKs, namely p38 and ERK, is a common event and has been shown to be important for cytokine production, as it is the case of IL-10 (1). For that reason, we asked if TLR2 or TLR4 driven MAPK phosphorylation would be different among *M. tuberculosis* H37Rv and *M. tuberculosis* Harlingen stimulations thereby influencing IL-10 mRNA stability. We found that *M. tuberculosis* Harlingen was able to induce p38 and ERK phosphorylation in a stronger and more prolonged fashion than *M. tuberculosis* H37Rv. Regarding LPS and Pam3Csk stimulation, TLR4 signaling induced more p38 and ERK phosphorylation but with a different kinetics from the one seen with the mycobacterial strains. This could be related to the fact that macrophages stimulation with mycobacteria activates other receptors besides TLR2 and TLR4 (36, 37) possibly contributing for an earlier MAPK phosphorylation. Interestingly, the inhibition of ERK had no influence on IL-10 mRNA degradation in response to *M. tuberculosis* Harlingen and LPS suggesting that ERK does not have a role in this post-transcriptional mechanism for IL-10 regulation. However, ERK inhibition resulted in lower amounts of IL-10 production, in accordance to previous reports (27, 29, 32, 33). Importantly, IL-10 mRNA degradation in response to *M. tuberculosis* Harlingen and LPS was greatly increased upon p38 inhibition which translated into significant lower amounts of cytokine production. In line with this, previous studies show that, in the context of single TLR stimulation, p38 inhibition is detrimental for IL-10 production (29-31). Following TLR engagement several signaling pathways, such as p38 and ERK phosphorylation, are initiated upon activation of the adaptor molecules MyD88 and TRIF (25, 26, 38) leading to the production of pro-inflammatory cytokines, but also IL-10 (27, 28, 53). However, in the context of TLR2 and TLR4 signaling pathways, the precise contribution of MyD88 (recruited by both TLR2 and TLR4) and TRIF (recruited only by TLR4) (25, 26) for the post-transcriptional regulation of IL-10 was never addressed before. Here we showed that in response to TLR4 signals, TRIF mediated the prolonged p38 phosphorylation and consequently the stabilization of the IL-10 mRNA. The ability of TLR4 derived TRIF activation to sustain p38 phosphorylation has been shown before (54, 55), however, our work for the first time links the TRIF-dependent p38 phosphorylation with protection of the IL-10 mRNA from rapid degradation. Thus, we now provide a molecular mechanism by which TRIF regulated IL-10 production.

Importantly, we were also able to confirm this novel pathway in the context of *Escherichia coli* stimulation (Supplementary Fig. 1), which not only further validates our results, but also strongly suggests that the IL-10 post-transcriptional mechanism identified in our study is conserved among pathogens that strongly activate TLR4.

A missing link of the work presented here is how p38 protects the IL-10 mRNA from degradation. p38 is reported to be key for IL-10 mRNA stabilization protecting it from the destabilization action of tristetraprolin (TTP) (34). TTP is a zinc finger protein identified as RNA binding - molecule that targets containing class II adenosine-uridine-rich elements (AREs) on the 3´untranslated region (UTR) of many cytokines, signaling them for rapid mRNA degradation (56). Moreover, in response to LPS, TTP-/- macrophages show decreased IL-10 mRNA degradation rates and increased IL-10 production (51). Considering these reports, we are currently assessing the contribution of TTP to our system. We anticipate that, as compared to TLR2/MyD88 signals (*M. tuberculosis* H37Rv and Pam3Csk), TTP activation will be more efficiently counteracted by strong and prolonged p38 phosphorylation in response to signals mediated by TLR4/TRIF (*M. tuberculosis* Harlingen and LPS).

In summary our work unveils a novel layer of post-transcriptional regulation of IL-10 that associates TLR triggering, the TRIF adaptor molecule and p38 phosphorylation. Since increased IL-10 production has been considered as detrimental during the course of *M. tuberculosis* experimental infection (5, 17), shown to be associated with patients with active disease (19, 21, 22) and increased in response to hyper-virulent strains, as compared to less virulent strains (57, 58), our study also highlights the possibility of TLR4 triggering as a virulence factor of *M. tuberculosis* and other pathogens, through IL-10 production.

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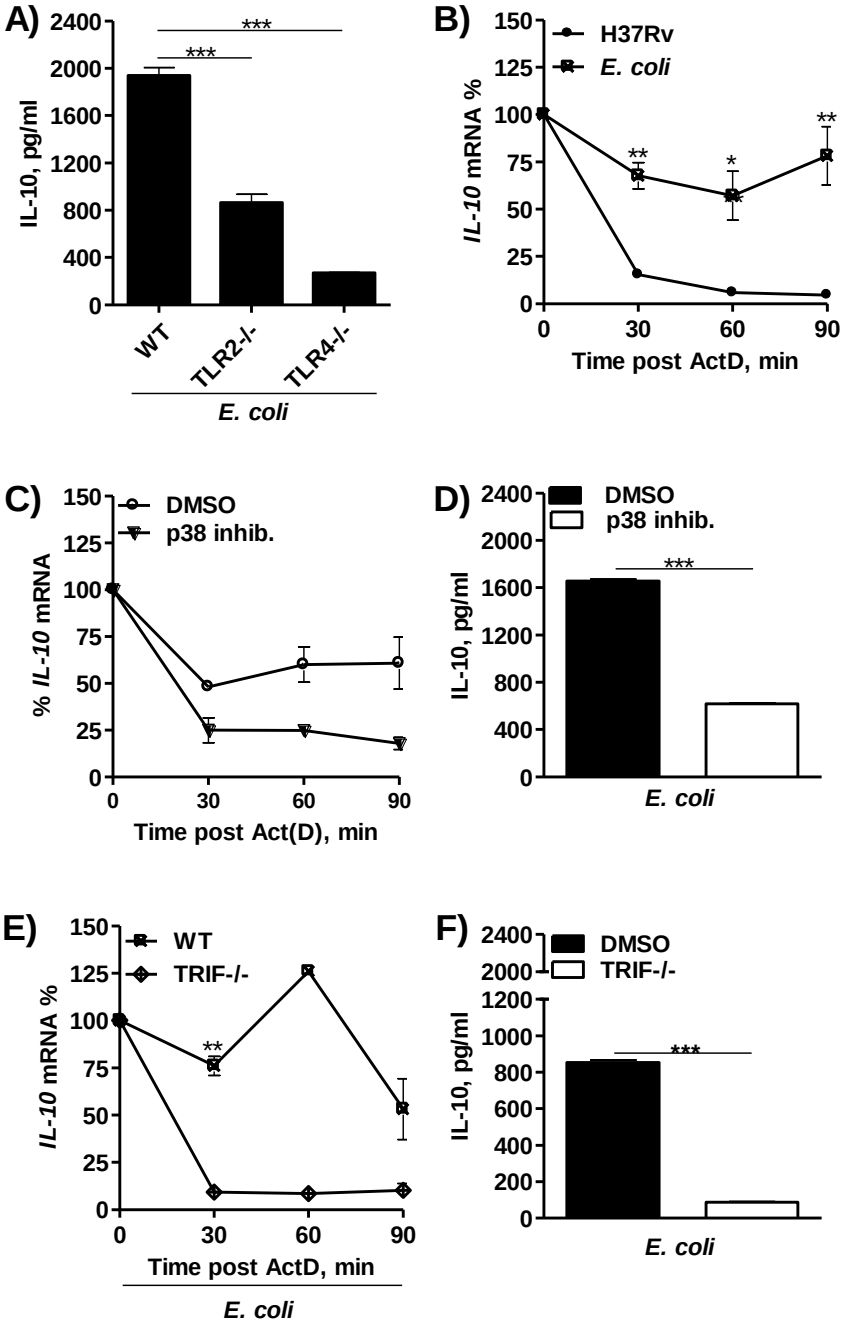
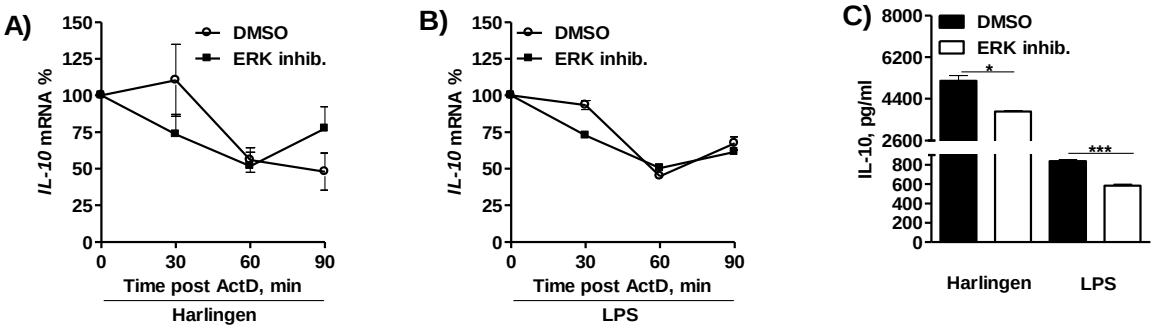
Supplementary Figure 1



Supplementary Figure 1. WT, TLR2-/- , TLR4-/- and TRIF-/- macrophages were stimulated with *E. coli* and (A – F) and *M. tuberculosis* H37Rv (B).When indicated, a p38 inhibitor was added to cell cultures 50 min post stimulation and ActD added 1hr post stimulation. (A, D and F) 6hrs post infection cell supernatants were collected and the amount of IL-10 assessed by ELISA. (B, C, E) At the indicated time points post ActD, RNA was extracted, converted to cDNA and the expression of *IL-10* was analyzed by RT-PCR. Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the

Student´s t test (*p<0.05; ***p<0.001). The data are representative of three (A-C) and one (D-E) independent experiments.

Supplementary Figure 2



Supplementary Figure 2. WT and TRIF-/- macrophages were stimulated with *M. tuberculosis* Harlingen and LPS and when indicated, in the presence, or absence, of an ERK inhibitor added 50 min post stimulation. (A, B) At the indicated time points post ActD addition to cell culture RNA was extracted and the expression of IL-10 was analyzed by RT-PCR. (C) Cell culture supernatants were collected 6hrs post stimulation and the amount of IL-10 measured by an ELISA assay.. The data are representative of three independent experiments. Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the Student´s t test (*p<0.05; ***p<0.001).

CHAPTER 3.2

Higher IL-10 and lower IL-12 production by dendritic cells are induced upon stimulation with *Mycobacterium bovis* BCG as compared to *Mycobacterium tuberculosis*.

Higher IL-10 and lower IL-12 production by dendritic cells are induced upon stimulation with *Mycobacterium bovis* BCG as compared to *Mycobacterium tuberculosis*.

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ABSTRACT

Mycobacterium tuberculosis (Mtb) accounts for approximately 2 million deaths per year, according to the World Health Organization. The current vaccine against Mtb infection, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), is a live attenuated strain of *M. bovis*. *M. bovis* BCG protects against tuberculous meningitis in infants, but it shows great variability in the prevention of Tuberculosis (TB) in adults. Studies on how this vaccine functions, including on how the molecular immune response triggered by *M. bovis* BCG compares to that of Mtb, are of importance for improving *M. bovis* BCG efficacy. A crucial step for *M. bovis* BCG-induced protection is the timely differentiation of T helper (Th) cell responses, particularly of Th1 cells. The differentiation of Th1 cells is highly dependent on the production of IL-12 by dendritic cells (DCs), which is in turn regulated by many factors, including IL-10. Therefore, IL-10 may play a detrimental role in vaccination by *M. bovis* BCG due to its role in down-modulating the immune response. In this study, we investigated the molecular mechanisms that regulate IL-10 expression by DCs upon *M. bovis* BCG stimulation as compared to Mtb stimulation. We found that, as compared to Mtb, *M. bovis* BCG induced higher expression of IL-10 by bone marrow derived DCs (BMDC). Our study also showed that Mtb and *M. bovis* BCG are differently recognized by TLR2 and 9 and that IL-10 is a strong regulator of IL-12 responses. Overall, our work shows that *M. bovis* BCG recognition by TLR2 is a major pathway for induction of IL-10 and inhibition of IL-12, in what compares to Mtb. Our future goal is to understand the molecular basis leading to the differential IL-10 production by *M. bovis* BCG- *versus* Mtb-stimulated DCs. The dissection of the molecular events involved in the differential responses to *M. bovis* BCG *versus* Mtb infection could provide further insights on how these bacteria interact with the immune system.

INTRODUCTION

Infection with *Mycobacterium tuberculosis* (Mtb) remains nowadays a threat to public health leading to high rates of morbidity and mortality (1, 2). *M. bovis* bacillus Calmette-Guérin (BCG), the only vaccine in use against tuberculosis (TB), is of limited value to prevent adult TB (3, 4). The reasons as to why *M. bovis* BCG induces variable protection against Mtb are not completely elucidated, so the study of the cellular and molecular mechanisms generated in response to *M. bovis* BCG as compared to the ones driven by Mtb is a good starting point to identify pathways and key molecules that can help to optimize *M. bovis* BCG as a vaccine.

Dendritic cells (DCs) are essential for the initiation and regulation of adaptive immune responses to various stimulus (5, 6), namely mycobacteria (7-9). The initial recognition of mycobacteria by DCs occurs via different pattern recognition receptors, such as Toll like receptors (TLRs) 2 and 9, triggering the production of cytokines like IL-12 and IL-10 (10-15). The ability of DCs to produce IL-12 in response to mycobacteria is essential for the differentiation of T helper (Th)1 CD4+ T cell responses (16-19). The immune control of Mtb infection is highly dependent on the production of IL-12 and generation of IFN-γ producing Th1 CD4+ T cells (19-25) and, furthermore, in humans a major role in protection afforded by IFN-γ (26, 27) and IL-12 has been established (28, 29). IL-10, an important anti-inflammatory cytokine, has been implicated in the inhibition of numerous protective immune responses such as macrophage microbicidal mechanisms (30), T cell proliferation and IL-12/IFN-γ production, both in response to mycobacteria or other types of stimulation (31-34). However, the role of this cytokine in the immune response against mycobacteria is still controversial, as different studies using mice models that lack IL-10 show either no role in protection against infection or increased susceptibility (35, 36). Importantly, in the context of *M. bovis* BCG vaccination, IL-10 seems to be involved in the generation of impaired protective responses to *M. tuberculosis* (17). Collectively, more detailed studies are necessary to fully understand the part that IL-10 takes upon infection and vaccination.

In the present work, we proposed to study the molecular mechanisms that regulate IL-10 expression upon the recognition of *M. bovis* BCG by bone marrow derived DCs (BMDCs) and how they relate to those in place upon Mtb recognition. We also studied the impact of IL-10 in IL-12 production by *M. bovis* BCG- versus Mtb- infected BMDCs.

MATERIAL AND METHODS

Mice. All animals used were females on the C57BL/6 background with eight to twelve weeks of age. Wild type (WT) and IL-10 deficient (-/-) mice were purchased at Charles River (Barcelona, Spain).TLR2 and TLR9 were a kind gift of Dr. Shizuo Akira, and kept in our animal facilities. Mice were used according to the European Union Directive 86/609/EEC and to the national authority, Direcção Geral de Veterinária.

Experimental infection. The H37Rv strain of Mtb and *M. bovis* BCG Pasteur were grown in Proskauer - Beck medium containing 0.05% Tween 80 to mid–log phase and frozen in 1 ml aliquots at –70°C. In all assays, mycobacteria were used at a multiplicity of infection of two.

Reagents. IFN-γ was used at 100U/ml and obtained from R&D Systems. The ERK inhibitor (U0126) was used at 2,5 μM and obtained from Calbiochem.

Generation of BMDCs. Cells were extracted from femurs and tibiae and cultured in DMEM (Dulbeco´s modified Eagle´s medium - GIBCO) supplemented with 10% of fetal bovine serum 1% sodium pyruvate, 1% hepes and 1% L-glutamine (GIBCO) and 20% of J558-cell conditioned media (GM-CSF), as a BMDCs driving factor. Briefly, 5 ml of cells ($1x10^6$ c/ml) were plated per well in a 6 well plate. By day 2 the cells culture media was replaced and wells gently washed to remove non-adhering cells. At day 4 the culture media was once more replaced without washing the wells and at day 6 non- adhering cells were collected counted and replated at a concentration $0,5x10^6$ c/ml. By day 7 of culture, non- adhering cells were once more collected, replated at $1x10^6$ c/ml in 24 well plates and infected. The cultures were maintained under 37°C and 5% CO_2 conditions for throughout the BMDCs generation and infection assays.

Protein detection. Cell culture supernatants were collected at the indicated time points and kept at - 80 °C until further analysis. The concentrations of IL-12p40, IL-12p70 and IL-10 were assessed by using a quantitative ELISA kit and performed according to the manufacturer (eBioscience).

RNA extraction, cDNA and quantitative real time PCR. Total RNA from stimulated and non-stimulated cell culture samples was extracted with TRIzol® 143 Reagent (Invitrogen, San

Diego, CA) according to the manufacturer's instructions. cDNA was synthesized using a reverse transcriptase reaction according to the manufacture (Fermentas) and on the MyCycler™ thermal cycler (Bio-Rad). Using quantitative reverse transcription polymerase chain reaction (RT-PCR), the relative gene expression of IL-12p40 was assessed with the SYBR Green Supermix, as recommended by the company (Fermentas), and performed using the CFX96™Real-Time System (Bio-Rad). Ubiquitin was the chosen reference gene. Both Ubiquitin and IL-10 were purchased at Invitrogen: ubiquitin forward, 5´ - GCTGGTGAAAAGGACCTCT - 3´; ubiquitin reverse, 5´ - CACAGGACTAGAACACCTGC - 3´; IL-10 forward 5'- TTT GAA TTC CCT GGG TGA GAA -3'; and IL-10 reverse 5'- GCT CCA CTG CCT TGC TCT TAT T -3'; IL-12p40 forward, 5'- CAA ATT ACT CCG GAC GGT TCA -3'; IL-12p40 reverse, 5'- AGA GAC GCC ATT CCA CAT GTC -3'.

Statistical analysis. The data was obtained from independent experiments, expressed as Mean ± SEM and analyzed using the two-tailed Student´s t test. The p values considered as having statistical significance were 0, 05≤p*, 0, 01≤p** and 0, 001≤p***.

RESULTS AND DISCUSSION

***M. bovis* BCG is a stronger inducer of IL-10 expression by BMDCs than *M. tuberculosis*.** To investigate the molecular mechanisms underlying the regulation of IL-10 expression by Mtb- or *M. bovis* BCG-infected BMDCs, we firstly assessed their IL-10 production upon infection. We show that both Mtb and *M. bovis* BCG induced IL-10 production by BMDCs, however *M. bovis* BCG was a stronger IL-10 inducer (Fig. 1A). The potential to induce IL-10 by BMDCs shown in our study is in agreement with previous studies where *M. bovis* BCG (18, 37) and Mtb (13, 16, 38) were shown to induce IL-10. We next investigated whether these two mycobacteria were being recognized by the same TLRs or not and whether that impacted IL-10 production. For that, BMDCs from wild type and TLR2 or 9 deficient mice were infected, with either Mtb or *M. bovis* BCG, and the levels of cytokine production (and mRNA expression shown on supplementary Fig. 1 A-C) were measured.

Figure 1

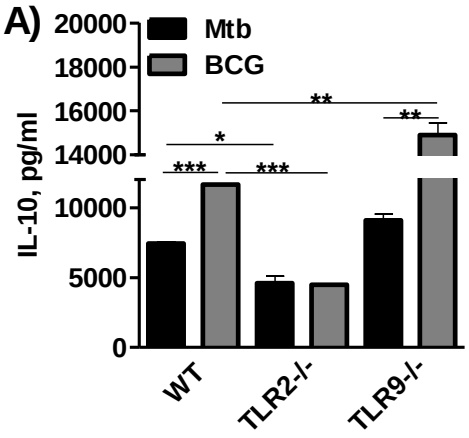

Figure 1. *M. bovis* BCG is a strong inducer of IL-10, as compared to Mtb. TLR2 and TLR9 are triggered differently in response to mycobacteria for the production of IL-10. WT, TLR2-/- and TLR9-/- BMDCs were infected with Mtb or *M. bovis* BCG (MOI of 2). The amount of IL-10 in the culture supernatants was measured by ELISA, 24 hrs post infection. Results are Mean ± SEM value of 3 replicates per group and the p values were determined by the Student´s t test (*p<0.05; **p<0.01; ***p<0,001). The data are representative of 3 independent experiments.

As shown in Fig. 1A, in response to either Mtb or *M. bovis* BCG the amount of IL-10 secreted by BMDCs was reduced in the absence of TLR2 signaling as compared to WT cells. Our data are in line with previous studies showing that IL-10 production by antigen presenting cells (APCs) is greatly impaired in the absence of TLR2 upon exposure to mycobacterial antigens (12, 15), which favors pro-inflammatory responses such as IL-12 production (15). Interestingly, in response to *M. bovis* BCG, IL-10 production was also partially inhibited by TLR9 triggering, since in the absence of this TLR there was a considerable increase of IL-10. In fact, although TLR9 has been shown to be able to promote IL-10 production (39, 40), some studies show that, either *in vivo* (10) or in *vitro* (10, 11, 39, 41), TLR9 has a great potential for positively modulate Th1 responses, namely by inducing IL-12 production in the context of mycobacterial infections. Considering the negative role of IL-10 in promoting a protective immune response to *M. bovis* BCG vaccination (17) and our results showing a strong IL-10 production by BMDCs in response to Mtb and, especially, *M. bovis* BCG we next hypothesized that in our model the IL-12 responses induced by *M. bovis* BCG on BMDCs would be compromised as compared to the ones driven by Mtb.

IL-12p70 inhibition by IL-10 is stronger in response to *M. bovis* BCG as compared to Mtb. The important role of IL-12 in driving protective CD4+ Th1 responses against mycobacteria has been demonstrated experimentally (16, 21, 22, 42-44) and further supported by increased susceptibility to TB in humans with genetic defects on IL-12 or IL-12 receptor genes (45, 46). Bearing in mind the protective role of IL-12 in mycobacterial infections and considering the above results in which *M. bovis* BCG led to higher IL-10 production than Mtb in infected BMDCs, we aimed at comparing the BMDCs-IL-12p70 response to Mtb and *M. bovis* BCG, both in the presence and absence of endogenous IL-10. For this, WT and IL-10 deficient BMDCs were infected with either Mtb or *M. bovis* BCG and supernatants were collected after 24 hrs. Fig. 2A shows that the amount of IL-12p70 produced by BMDCs was significantly lower in response to *M. bovis* BCG when compared to Mtb, possibly due to a higher IL-10 production profile (Fig. 1A). Notably, in the absence of endogenous IL-10 both mycobacteria induced increased IL-12p70 production by BMDCs, being the amount of this cytokine closer between these two conditions than it was in the WT situation. The ability of IL-10 in dampening IL-12 transcription and production has been reported before in other experimental set ups (47, 48). Furthermore, it has been shown the inverse correlation between IL-10 and IL-12 induction in response to

mycobacterial antigens (15) and, additionally, in response to whole Mtb or *M. bovis* BCG, where the production of IL-12p70 is greatly enhanced in BMDCs when IL-10 is absent (18, 38). What we now show is that the levels of IL-10 induced by *M. bovis* BCG are higher than those induced by Mtb, with an important impact on IL-12 production.

Figure 2

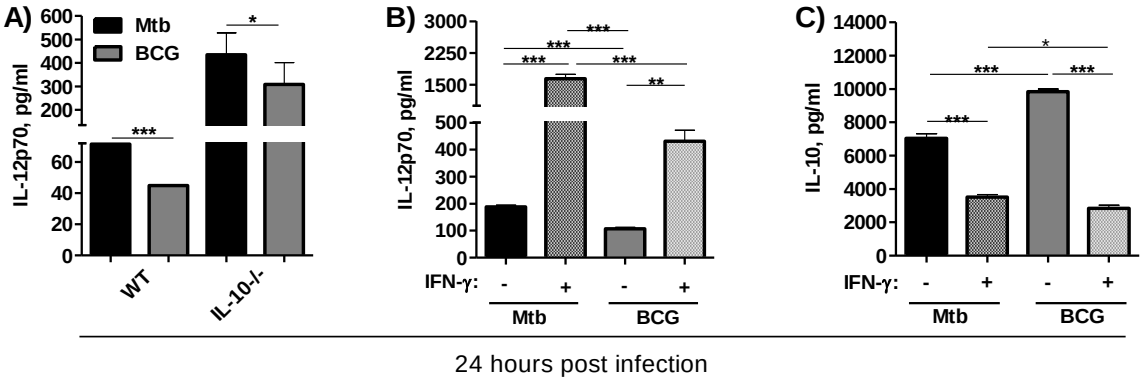

Figure 2. IL-10 is a key regulator of IL-12 production by mycobacteria infected BMDC. IFN-γ positively regulates IL-12 and negatively regulates IL-10. Wild type and IL10-/- BMDCs were infected with Mtb or *M bovis* BCG (MOI of 2) (A-C). When indicated, IFN-γ was added to the wild type cell cultures together with the mycobacteria (B-C). The amounts of IL-12p70 (A and B) and IL10 (C) in response to mycobacteria were measured by an ELISA. Results are mean ± SEM value of 3 replicates per group and the p values were determined by the Student´s t test (*p<0.05; **p<0.01; ***p<0,001). The data are representative of three independent experiments.

We next investigated if the presence of IL-10 would turn the infected cells, especially the ones infected with *M. bovis* BCG, somewhat impaired and possibly unresponsive to external signals that otherwise would increase IL-12 production. To test this hypothesis BMDCs cultures were infected with Mtb and *M. bovis* BCG and supplemented, or not, with IFN-γ. Fig. 2B shows that both mycobacteria were able to induce increased amounts of IL-12p70 by BMDCs in the presence of IFN-γ, yet the IL-12p70 production induced by *M. bovis* BCG still was greatly impaired as compared to Mtb. However, Fig. 2C shows that the presence of IFN-γ inhibited IL-10 production resulting in comparable levels in response to Mtb and *M. bovis* BCG. Since in the presence of IFN-γ the amount of IL-12p70 produced by *M. bovis* BCG-infected BMDCs is still lower than that induced upon Mtb infection, our data suggest that other molecules besides IL-10

may contribute for the IL-12p70 differences observed between these two mycobacteria. Taken together, our data show that despite early and consistent IL-10 production by BMDCs in response to mycobacterial challenges, cells are still able to be further activated by external signals such as IFN-γ. The addition of IFN-γ to the cell culture positively and negatively regulated IL-12 and IL-10 production, respectively, which is in line with reports showing that IFN-γ is able to block IL-10 (38, 49, 50) and also to enhance IL-12 production (11, 38).

Collectively, our observations suggest that BMDCs produce IL-10 in response to *M. bovis* BCG and consequently are limited in their IL-12 production, which might help to understand why *M. bovis* BCG has a limited efficacy as a vaccine. In agreement with a role for IL-10 in limiting *M. bovis* BCG efficacy, *Gopal* and colleagues (17) have shown recently that *M. bovis* BCG vaccination promotes better protection against Mtb in IL-10 -/- mice as compared to WT animals. Notably, IL10-/- BMDCs infected with *M. bovis* BCG and transferred into mice are able to increase the IFN-γ response in the draining lymph nodes as compared to WT BMDC (18). To further support the idea that IL-10 impacts the differentiation of Th1 cells during mycobacterial infection, Redford *et al* (51) showed that, IL-10-/- mice are more resistant to a primary infection presenting lower bacterial burdens and increased IFN-γ and CD4+ Th1 responses in the lung. We are currently testing how IL-10 impacts the ability of BMDCs to generate Th1 cells in response to *M. bovis* BCG in what compares to Mtb by taking advantage of the OT-II transgenic mouse model.

Mycobacterial infection regulates IL-12p40 in a negative and positive fashion via TLR2 and TLR9, respectively. Along with IL-12p70, the $(p40)_2$ homodimer has been shown to be implicated in the generation of protective immune responses in response to Mtb as it is essential for the migration of DCs from the periphery to the DLNs, where the generation of protective Th1 cells are generated (16). Furthermore, although protective immune responses become compromised in the absence of IL-12p70, IL-23 (heterodimer composed by p19 and p40 subunits) has been shown to be required for a moderate level of protection when IL-12p70 is compromised (52). Moreover, in the absence of the $(p40)_2$, upon mycobacterial exposure, DCs have an impaired capacity to migrate from the lung to the lymph node, being enable to drive Th1 type of responses (16). Taking into consideration the fact that IL-10 limits $(p40)_2$ production, thereby compromising the migration of DCs to the draining lymph nodes (16, 18), we questioned if, in our model, IL-12p40 would be differently induced by Mtb and *M. bovis* BCG and if TLR2 and 9 would have an impact in that response. In Figure 3A-B we show that, as seen before for IL-

12p70 (Fig. 2A), *M. bovis* BCG was a weaker inducer of IL-12p40 as compared to Mtb. Also, in response to either mycobacteria, the absence of TLR2 resulted in an increased IL-12p40 production by BMDC, when compared to WT cells (Fig 3A). In line with these results, triggering via TLR2 has been shown to activate signaling pathways that are detrimental for IL-12 production (53, 54), resulting in the low production of this cytokine. In contrast, TLR9 triggering seems to be important for IL-12p40 induction since in its absence the production of this cytokine production was diminished (Fig. 3A). These findings were also observed at the transcriptional level (Fig. 3B-D), which fits with findings showing that TLR9 signaling is important at driving IL-12 production (10, 11, 39, 41). Furthermore, in the absence of TLR2, Mtb was able to induce a higher peak of IL-12p40 transcription at 6 hrs, although this profile was not sustained overtime (Fig. 3C). In contrast, *M. bovis* BCG induced lower but sustained IL-12p40 transcription by DCs (Fig. 3D). Differences in IL-12p40 mRNA sustained profile could be due to a higher rate of IL-12p40 mRNA degradation in response to Mtb, when compared to *M. bovis* BCG, however further studies are needed to address this possibility.

Figure 3

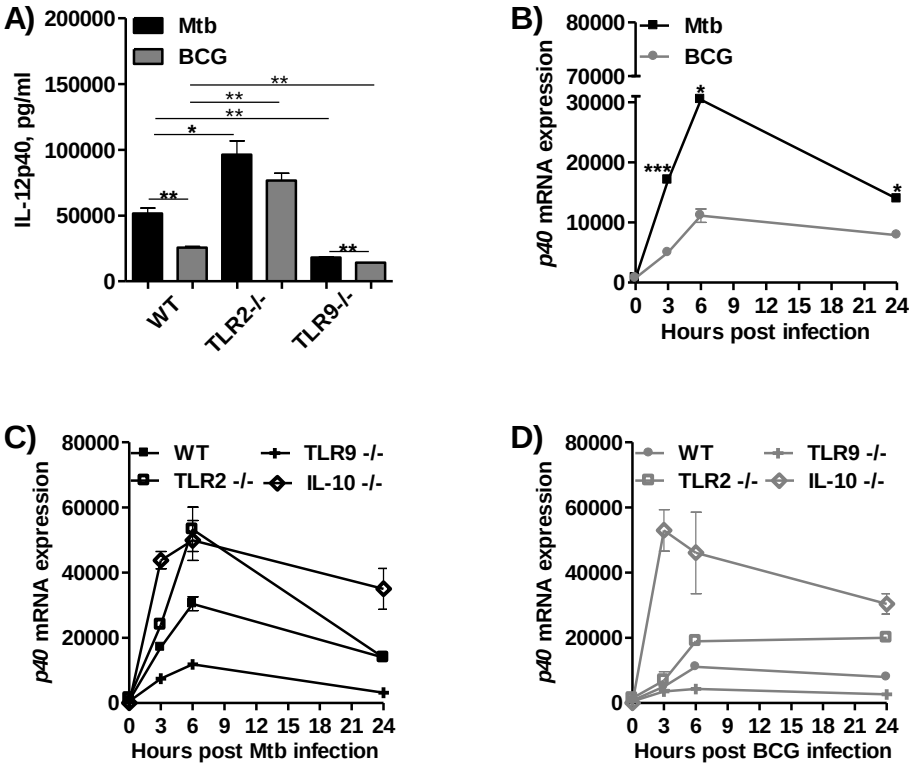

Figure 3.Mycobacteria regulates IL-12p40 in a negative and positive fashion via TLR2 and TLR9, respectively. WT, TLR2-/- and TLR9-/- BMDCs were infected with Mtb or *M. bovis* BCG (MOI of

2) (A-D). Cell culture supernatants were collected 24 hrs post infection and the amount of IL-12p40 was measured by ELISA (A). At the indicated time points post infection RNA was extracted and the expression of *p40* was analyzed by quantitative real-time PCR and normalized to the expression of ubiquitin (B–D). Results are Mean ± SEM value for 3 replicates per group and the p values were determined by the Student´s t test (*p<0.05; ***p<0.001). The data are representative of 3 independent experiments.

In response to mycobacteria, ERK signaling differently regulates IL-12p70 and IL-10 production. Since TLRs trigger pathways such as mitogen activated protein kinases (MAPKs) signaling for cytokine induction (40, 55), we next hypothesized if the MAPK ERK was responsible for the different levels of cytokine production induced by Mtb and *M. bovis* BCG. To test this WT BMDC were infected with Mtb or *M. bovis* BCG in the presence or absence of an ERK inhibitor and the amounts of IL12p70 and IL-10 production were assessed 24 hrs later. In Fig. 4A we show that blocking ERK signaling led to a substantial increase of IL-12p70 upon Mtb or *M. bovis* BCG stimulation of BMDCs. However, ERK inhibition was not enough to level the IL-12p70 production driven by *M. bovis* BCG to the one promoted by Mtb. As for IL-10, Fig. 4B indicates that in response to either mycobacteria, ERK signaling is necessary for a full production of IL-10. Since in the absence of ERK we still observed IL-10 production, additional pathways were certainly involved to promote this response. One of these is likely to be p38 that, from results in our lab, is important for IL-10 production (Teixeira-Coelho *et al*, in prep.).

Figure 4

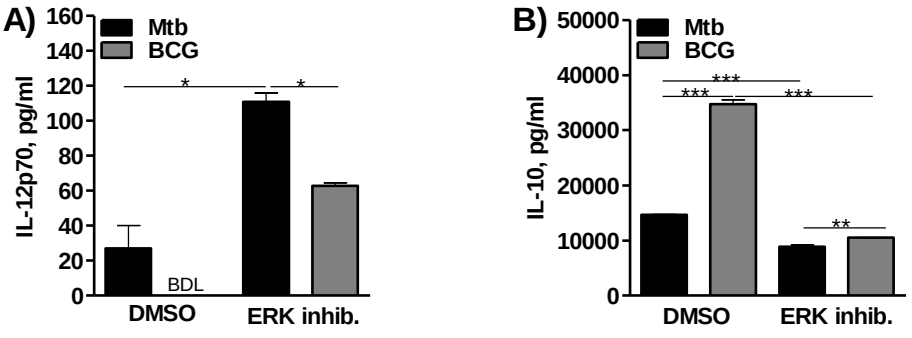

Figure 4. ERK signaling is involved in the inverse production of IL-12p70 and IL-10 in response to mycobacteria. WT BMDCs were infected with Mtb or *M. bovis* BCG (MOI of 2) and treated (or not) with an ERK inhibitor. Cell culture supernatants were collected 24 hrs post infection and the amount of IL-12p70 (A) and IL-10 (B) were measured by ELISA. Results are Mean ± SEM value for 3

replicates per group and the p values were determined by the Student´s t test (*p<0.05; ***p<0.001). The data are representative of 2 independent experiments.

In summary, in this part of the work, we have compared the ability of Mtb and *M. bovis* BCG to induce IL-10 and IL-12 production by BMDCs and found that, as compared to Mtb, *M. bovis* BCG induced higher amounts of IL-10 and lower of IL-12. For both mycobacteria full production of IL-10 was dependent on TLR2 activation, whereas IL-12 was negatively regulated by TLR2, thus suggesting that this TLR is of major importance in the regulation of the IL-10/IL-12 axis. Regarding the involvement of TLR9, we found that IL-12 production is highly dependent on this receptor while IL-10, at least in the case of *M. bovis* BCG, appeared to be negatively regulated. In line with our results, previous reports show that TLR9 has a greater ability, as compared to TLR2, to induce a stronger remodeling of the p40 promoter, thus resulting in higher IL-12p70 levels (11, 41). Furthermore, both TLR9 and TLR2 are able to activate the NF-kB pathway, very important for the production of pro-inflammatory cytokines, however TLR9 does it in a weaker and delayed fashion as compared to TLR2 which results in higher amounts of IL-12p70 (56). Alongside with these reports, IL-10 has also been shown to interfere with IL-12p40 transcription and resulting in impaired production of IL-12p70 production (47).

Altogether, we have been able to show that TLR9 is important for the production of IL-12 by BMDCs upon infection with mycobacteria and that the low potential of *M. bovis* BCG at inducing this cytokine is most likely related to the fact that this mycobacterium induces a strong IL-10 production. Importantly, as shown in Figure 1, in the absence of TLR2 there was still IL-10 production upon infection which indicates the involvement of other receptors in the triggering of IL-10. Moreover, in the absence of TLR2 the IL-10 levels induced were similar between *M. bovis* BCG and Mtb, which favors the hypothesis that *M. bovis* BCG might have TLR2 ligands that are present at higher frequency and diversity or that have more affinity to this TLR than those of Mtb. Regarding the involvement of ERK in response to *M. bovis* BCG or Mtb, we found that when this MAPK is inhibited IL-12 production is enhanced and IL-10 production is decreased. Moreover, in the absence of ERK signaling, IL12-p70 production is further augmented in response to TLR9 stimulation (40). Importantly, the ability of TLR2 to induce IL-12p40 is inhibited by ERK and cFOS, a downstream transcription factor targeted and stabilized by ERK and responsible for IL-10 induction and IL-12 inhibition in response to TLR2 signals (53, 54, 57, 58). It is conceivable that, as compared to Mtb, *M. bovis* BCG signaling through TLR2 is able to induce stronger ERK

phosphorylation and, consequently, stronger cFOS activation thus leading to an over-expression of IL-10 thereby potently inhibiting IL-12. Further studies need to be carried out to assess this hypothesis and also the contribution of another MAPK, p38, which has to been suggested by our group as a major regulator of IL-10 at the post transcriptional level (Teixeira-Coelho M *et al* in prep.).

Taking together the data presented in this chapter, in the context of Mtb and *M. bovis* BCG infected BMDCs, it would be interesting to further dissect: i) the contribution of receptors other than TLR for cytokine production, as it is the case of Dectin-1 that by activating ERK is able to induce IL-10 (57, 59); ii) the different TLR2 ligands present in *M. bovis* BCG and Mtb, their binding affinity relating that to IL-10 and IL-12 production and iii) the impact of IL-10/IL-12 balance in the differentiation of T helper types of response.

Further clarification of these aspects might help to the design of new preventive therapeutic approaches which relies on the deep understanding on how the pro and anti-inflammatory responses to mycobacterial infections are driven and how they correlate with protection. IL-10 regulation in the context of mycobacterial infections is thereby of major interest and has revealed to be a potential target of manipulation either in the context of primary infections (51) or vaccination set up (17).

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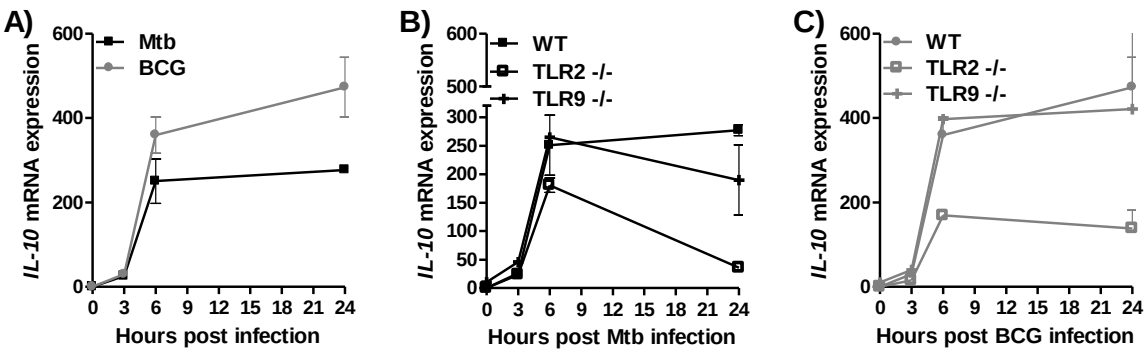
Supplemmentary Figure 1



Supplementary Figure 1. *M bovis* BCG induced IL-10 expression is stronger when compared to MTB. TLR2 is a key signal for IL-10 expression in response to mycobacteria. Wild type, TLR2-/- and TLR9-/- BMDCs were infected with *M. tuberculosis* or *M. bovis* BCG (MOI of 2) and at the indicated time points RNA was extracted. The expression of *IL-10* was analyzed by quantitative real-time PCR and normalized to the expression of ubiquitin (A-C). Results are Mean ± SEM value for 3 replicates per group. Data is representative of two independent experiments.

CHAPTER 3.3

TLR2 deficiency by compromising p19 (IL-23) expression limits Th17 cell responses to *Mycobacterium tuberculosis*

TLR2 deficiency by compromising p19 (IL-23) expression limits Th17 cell responses to *Mycobacterium tuberculosis*. Maria Teixeira-Coelho,[1,2] Andrea Cruz,[1,2] Jenny Carmona,[1,2] Carole Sousa,[1,2] Daniela Ramos-Pereira,[1,2] Ana Laura Saraiva,[1,2] Marc Veldhoen,[3] Jorge Pedrosa,[1,2] António G. Castro,[1,2] and Margarida Saraiva[1,2]. *International Immunology* (2010) Feb; 23(2):89-96. Epub 2010 Dec 14.

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TLR2 deficiency by compromising p19 (IL-23) expression limits T_h 17 cell responses to *Mycobacterium tuberculosis*

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Abstract

$CD4^+$ T_h1 cells producing IFN-γ are of extreme importance in controlling infections by *Mycobacterium tuberculosis* both in mice and in men. In addition to IFN-γ-producing T cells, IL-17-producing T cells (T_h17) have been observed during mycobacterial infections. Nevertheless, their contribution for the host immune response to mycobacteria as well as the signals triggering *M. tuberculosis* -specific T_h17 cell differentiation and maintenance are not fully understood. We show that signaling via Toll-like receptor (TLR) 2 has a major impact on the regulation of p19 (IL-23) expression in response to *M. tuberculosis* and therefore on the establishment of T_h17 cell responses to *M. tuberculosis* infection. Diminished T_h17 responses in the lung of *M. tuberculosis* -infected TLR2-deficient animals were not caused by defective cell differentiation in the draining lymph node (LN) but rather by reduced maintenance at the site of infection. Consistent with the decreased numbers of T_h17 cells in the lungs of infected TLR2-deficient animals, we observed reduced expression of CXCL9, CXCL10 and CXCL11, chemokines involved in recall responses to *M. tuberculosis*. Our data provides insights into the TLR2 role in infection with *M. tuberculosis*, with implications in pathophysiology of the disease and vaccine design.

Keywords: cytokines, IL-23, TLR, tuberculosis

Introduction

Phagocytic cells, such as macrophages, dendritic cells (DCs) and neutrophils, are among the first cells to sense the presence of *Mycobacterium tuberculosis* in the host. The recognition of *M. tuberculosis* by macrophages and DCs involves several pattern recognition receptors, such as Toll-like receptors (TLRs) (1, 2), DC-SIGN (3) and Dectin-1 (4). TLR2, 4 and 9 have all been shown to mediate the *in vitro* recognition of *M. tuberculosis* and the cytokine response of macrophages or DC to this pathogen is lower in the absence of these TLRs, particularly of TLR2 (5–12). As for the role of TLRs in the outcome of the infection by mycobacteria, mouse models of myeloid differentiation factor 88 (MyD88) deficiency showed that these animals are highly susceptible to experimental infections with *M. tuberculosis* (13–15) or with *Mycobacterium avium* (16), succumbing very early upon infection. In contrast, in experimental infections with low doses of *M. tuberculosis* inocula, TLR2-deficient (−/−) mice behave similarly to wild-type ones in terms of bacterial burden (7, 10, 12, 17, 18), although a defective granuloma formation was observed (7, 10, 17, 18). When high doses of pathogen were used (7, 18) or when the TLR2 deficiency was combined with TLR9 deficiency (10), a more pronounced role for TLR2 in the development of a protective immune response to *M. tuberculosis* was uncovered. In humans, several reports have linked the existence of polymorphisms in *TLR2* or TLR2 signaling molecules with susceptibility to *M. tuberculosis* (19–24) and *Mycobacterium leprae* (25). However, in some populations, TLR2 polymorphisms do not associate with increased risk of tuberculosis (26). In another study, a variant of the TLR signaling adapter protein Mal, showing an attenuated TLR2 signal transduction, was found to be protective against tuberculosis (27). Therefore, the association of *TLR2* polymorphisms with susceptibility to tuberculosis or severity of disease remains controversial and appears to greatly depend on the genetics of the host/bacteria interplay (28).

T-cell responses, in particular by IFN-γ-producing $CD4^+$ T_h1 cells, are of extreme importance in controlling infections by *M. tuberculosis* both in mice and in men (29, 30). TLR9, but not TLR2, was shown to participate in the generation of T_h1 cell responses in *M. tuberculosis*-infected mice, as in its absence less IFN-γ-producing $CD4^+$ T cells were observed (10). In contrast, a more recent study showed unaffected development of T_h1 cell responses in the absence of the master signaling adapter MyD88 or in the combined absence of TLR2/TLR4/TLR9 (12). In addition to IFN-γ-producing T cells, IL-17-producing T cells, both γδ T cells (31–33) and T_h17 cells (34–36), have been observed during mycobacterial infections. Although IL-17 appears to have a limited role in the host defense against this pathogen during primary aerogenic infection (37–39), IL-17A deficiency impacts both granuloma formation and bacterial burden (33). During vaccination against *M. tuberculosis*, T_h17 cells were described to be protective by accelerating the recruitment of T_h1 cells to the site of infection (40). Furthermore, de-regulated production of IL-17, due to repeated exposure to mycobacterial antigen, has been recently shown to associate with extensive lung pathology (41). In humans, an increased frequency of T_h17 cells was associated with latency (42), whereas in another study, reduced T_h17 cells were associated with more severe disease (43). Thus, the relative contribution of T_h17 cells remains to be elucidated in human *M. tuberculosis* infection and in animal models.

The recognition mechanisms and signals that lead to IL-17 production by γδ T cells or to T_h17 cell differentiation during infection by *M. tuberculosis* remain elusive. Martin *et al.* (44) reported that TLR2-mediated recognition of inactivated *M. tuberculosis* enhances IL-17 production by γδ T cells. A role for TLR9 in regulating *Mycobacterium bovis* bacille Calmette-Guérin elicited immune responses in mice, through the expression of delta-like four Notch ligand, was reported, with lower T_h17 responses observed in the absence of TLR9 (45). Furthermore, understanding these signals are important considering the role of IL-17 in vaccination, protection and pathology during mycobacterial infections. To address this issue, we investigated the role of TLR2 in the development of T_h17 cell responses to *M. tuberculosis*. We found that TLR2 deficiency strongly impacts p19 (IL-23) expression during *M. tuberculosis* infection. As a result, the absence of TLR2 compromised the maintenance of *M. tuberculosis*-specific T_h17 cells at the site of infection. Interestingly, the differentiation of T_h17 cell in the draining LNs of infected mice was not affected by the absence of TLR2. Clarifying the molecular events determining the regulation of IL-17 production by TLR2 during mycobacterial infections may provide new hints for the modulation of vaccination, protective responses and pathology.

Methods

Bacteria

H37Rv strain of *M. tuberculosis* was grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen in 1-ml aliquots at −80°C. Inactivated H37Ra strain of *M. tuberculosis* was purchased from Difco Laboratories.

Animals and experimental infection

Eight- to 12-week-old female C57BL/6 mice, obtained from Charles River (Barcelona, Spain), C57BL/6 OT-II/rag1−/− (OT-II) TCR transgenic, obtained from the National Institute for Medical Research or $TLR2^{-/-}$ mice, maintained at ICVS Life and Health Sciences Research Institute were used. Mice were anesthetized with Ketamine/Medetomidine and infected intranasally with *M. tuberculosis* H37Rv, resulting in a dose of ~100–200 colony-forming units (CFUs) delivered into the lungs. All mouse protocols were performed according to the European Union Directive 86/609/EEC, and previously approved by the national authority Direcção Geral de Veterinária.

Bacterial load determination

Groups of five to seven infected wild-type or $TLR2^{-/-}$ mice were killed by CO_2 asphyxiation at several time points after *M. tuberculosis* infection, the lungs were aseptically excised and homogenized in PBS. Serial dilutions of the lung homogenate were plated on nutrient 7H11 agar. CFUs were counted after 3 weeks of incubation at 37°C.

Cell preparation and culture

Lungs and mediastinal LN of infected animals were aseptically removed and cell suspensions were prepared as described previously (40). Lung and mediastinal LN cells were then used for ELISPOT, RNA analysis and flow cytometric analysis of CD4 or of GR1, as described before (34, 40).

Quantitative real-time PCR analysis

Total RNA from infected and non-infected lungs and mediastinal LN was extracted with TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA was synthesized and analyzed by real-time PCR as described previously (46). Target gene mRNA expression was quantified using SYBR green (Qiagen) and specific oligonucleotides (Supplementary Table I is available at *International Immunology* Online) and normalized to the ubiquitin mRNA levels or using specific primer probes (ABI) (Supplementary Table I is available at *International Immunology* Online) and the LightCycler-FastStart DNA Master Hybridization (Roche) and normalized to the hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA levels.

Histological and morphometric analysis

Caudal lobes of lungs from wild-type or $TLR2^{-/-}$-infected mice were inflated with 3.8% phosphate-buffered formalin, fixed for 1 week and embedded in paraffin. Sections of 4-μm thickness were stained with hematoxylin and eosin (H&E) for the inflammatory index score or used to detect inducible Nitric Oxide synthase (iNOS) by immunofluorescence with a goat anti-mouse antibody (M-19G from Santa Cruz biotechnology) and detected with Alexa Fluor 568-conjugated polyclonal rabbit anti-goat (Invitrogen). 4′, 6-Diamino-2-phenylindole hydrochloride was used to counterstain tissues and to detect nuclei. All H&E sections were blindly analyzed by an independent pathologist and individually scored for inflammation according to the following scale: 0, absent; 1, mild; 2, abundant; 3, severe. Similarly, a score

was attributed to the number of iNOS foci: 0, no foci; 1, 0–10 foci; 2, 10–20 foci; 3, 20–30 foci; 4, >30 foci.

Statistics

Data are expressed as mean ± SEM, and the significance of differences between two means was assessed with two-tailed Student's *t*-test. Differences were considered statistically significant if $P \leqslant 0.05$.

Table 1. Bacterial counts (mean ± SEM) in the lungs of wild-type or TLR2$^{-/-}$ mice infected with *Mycobacterium tuberculosis*[a]

	Bacterial counts (log_{10} CFUs)	
	Wild-type	TLR2$^{-/-}$
Day 1	2.28 ± 0.05	2.10 ± 0.11
Day 50	5.17 ± 0.42	4.71 ± 0.15
Day 215	5.33 ± 0.30	4.69 ± 0.28

[a]At the indicated time points, lungs from five to six infected mice were harvested and homogenized, diluted and then plated to determine the number of mycobacterial CFUs per organ. Means were determined to be not statistically different as described in Methods.

Results

TLR2 regulates T_h17 cell responses to infection with M. tuberculosis

To clarify the molecular signals determining the establishment of *M. tuberculosis*-specific T_h17 cell responses, wild-type or TLR2$^{-/-}$ animals were infected intra-nasally with a low dose of *M. tuberculosis*. The profile of T_h cell responses was analyzed by ELISPOT in the lungs of infected animals 21, 54 or 215 days post-infection. In line with previous reports (7, 10, 12, 17, 18), the bacterial burden in the lung of wild-type or TLR2$^{-/-}$ animals was similar (Table 1). The number of T_h17 cells, already seemingly reduced at 21 days post-infection, was significantly diminished at days 54 and 215 after *M. tuberculosis* infection in the absence of TLR2 (Fig. 1A). In contrast, the number of T_h1 cells appeared to be unaffected by the absence of TLR2 until very late time points post-infection (Fig. 1B). We also followed the expression of IL-17A and IFN-γ in the lungs of infected animals by real-time PCR. The expression of

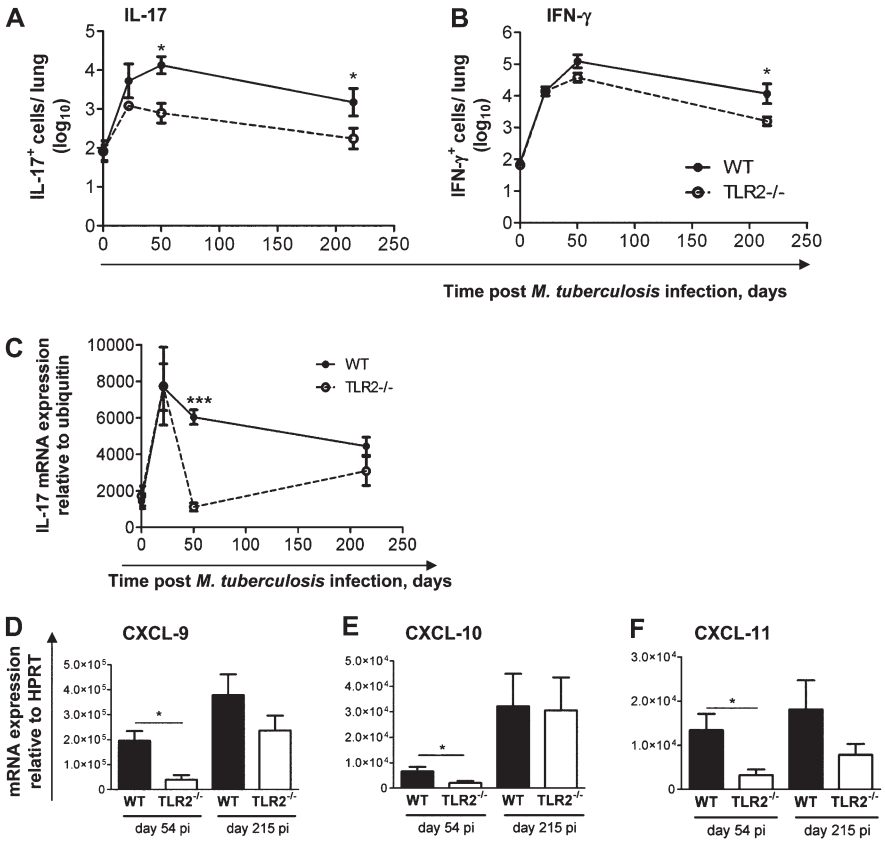

Fig. 1. Lung T_h17 cell responses during infections by *Mycobacterium tuberculosis* are compromised in the absence of TLR2. Wild-type or TLR2$^{-/-}$ mice were infected intra-nasally, and at the indicated time points, lung cell suspensions were prepared, isolated cells stimulated for 24 h with ESAT-6 peptide presented by total spleen irradiated antigen-presenting cells and IL-17 (A) or IFN-γ (B) production assessed by ELISPOT. In parallel, RNA was extracted from the lung tissue and the expression of IL-17 (C), CXCL9 (D), CXCL10 (E) or CXCL11 (F) analyzed by quantitative real-time PCR and normalized to the expression of ubiquitin (C) or HPRT (D–F). Data represented for day 0 correspond to uninfected animals. Data points show the mean ± SEM value for five to six mice per group and the significance was determined by the Student's *t*-test (*$P < 0.05$, **$P < 0.01$). The data are representative of two independent experiments.

IL-17 (Fig.1C), but not IFN-γ (data not shown), was highly affected by the absence of TLR2, being this effect more pronounced on day 54 post-infection. Consistent with the lower expression of IL-17, the transcription of chemokines known to be induced by IL-17 (40), namely CXCL9, CXCL10 and CXCL11 (Fig. 1D–F), was also diminished in the lung on day 54 post-infection. Our data thus suggest that TLR2 is an important upstream molecule in mediating T_h17 cell responses to *M. tuberculosis*.

M. tuberculosis-induced p19 expression is mediated by TLR2 signaling

Considering the importance of IL-23 for the establishment of T_h17 cells, we investigated whether IL-23 expression was modulated by TLR2 recognition of *M. tuberculosis*. We measured the expression of p40 and p19, the monomers that form bioactive IL-23, in the lungs of wild-type or TLR2$^{-/-}$ mice during the course of infection. We observed that the induction of p19 expression observed in wild-type mice did not occur in the lungs of *M. tuberculosis*-infected TLR2$^{-/-}$ mice (Fig. 2A). In addition, the overall transcription of p40 in the lungs of infected animals was lower in the absence of TLR2 (Fig. 2B), whereas the expression of p35, which together with p40 forms IL-12, was not significantly affected in the absence of TLR2 (Fig. 2C). The expression of IL-6 and IL-1β in the lungs of TLR2$^{-/-}$-infected animals was also significantly lower than in wild-type animals at early time points post-infection (Fig. 2D and E). Of note, since only the cleaved IL-1β molecule is biologically active, the fact that its mRNA is down-regulated in the absence of TLR2 should be interpreted with caution and only provides an indication that the amount of IL-1β may be decreased. Our data suggest that TLR2 is required for the induction of p19 and IL-1β expression, two key molecules for the establishment of appropriate *M. tuberculosis*-specific T_h17 responses.

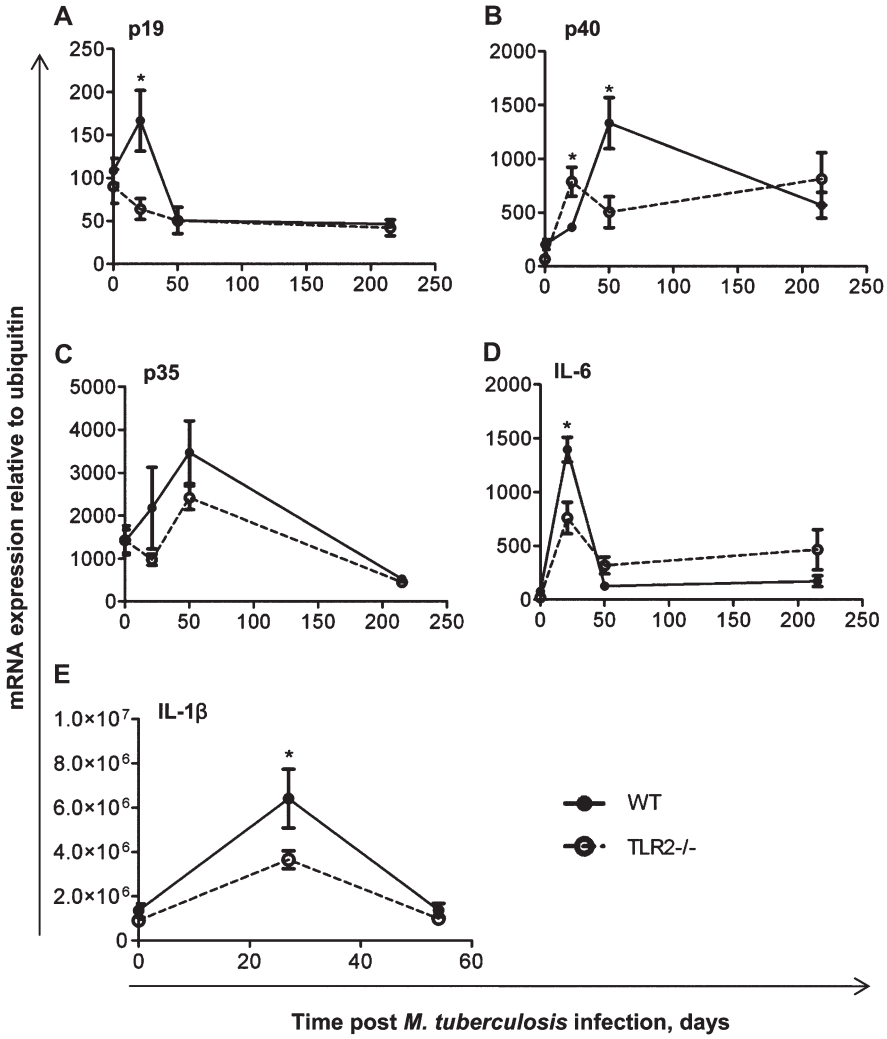

Fig. 2. TLR2 regulates the expression of p19 in the lung, during *Mycobacterium tuberculosis* infections. Wild-type or TLR2$^{-/-}$ mice were infected as before and at the indicated time points, lung tissue was obtained, RNA extracted and p19 (A), p40 (B), p35 (C), IL-6 (D) and IL-1β (E) expression measured by real-time PCR and normalized to ubiquitin. Data points show the mean ± SEM value for five to six mice per group and the significance was determined by the Student's *t*-test (*$P < 0.05$). Data represented for day 0 correspond to uninfected animals. The data are representative of two independent experiments.

TLR2 signaling during M. tuberculosis infection does not impact T_h17 cell differentiation in the LN

Taking into consideration that IL-23 is not involved in the initial steps of T_h17 cell differentiation (47), we next investigated whether the differentiation of T_h17 cells in the draining mediastinal LN during *M. tuberculosis* infection occurred normally in TLR2$^{-/-}$ mice. To address this issue, we started by comparing the total cell number (Fig. 3A) and the number of CD4$^+$ T cells (Fig. 3B) in the mediastinal LN of wild-type and TLR2$^{-/-}$ mice infected with *M. tuberculosis* for 14 or 28 days and found no differences. We chose these early time points since T_h cell differentiation upon *M. tuberculosis* infection has been described to start in the mediastinal LN at around day 14 post-infection (48, 49). We next assessed by ELISPOT, the number of T_h17 cells in the mediastinal LN of wild-type or TLR2$^{-/-}$-infected animals. As shown in Fig. 3(C), the number of IL-17-producing T cells in the mediastinal LN of infected animals was similar in the presence or absence of TLR2. Consistent with unaffected initiation of T_h17 responses in TLR2$^{-/-}$ mice, the expression of IL-6, although barely detectable in infected mediastinal LN, was not decreased in the absence of TLR2 (data not shown).

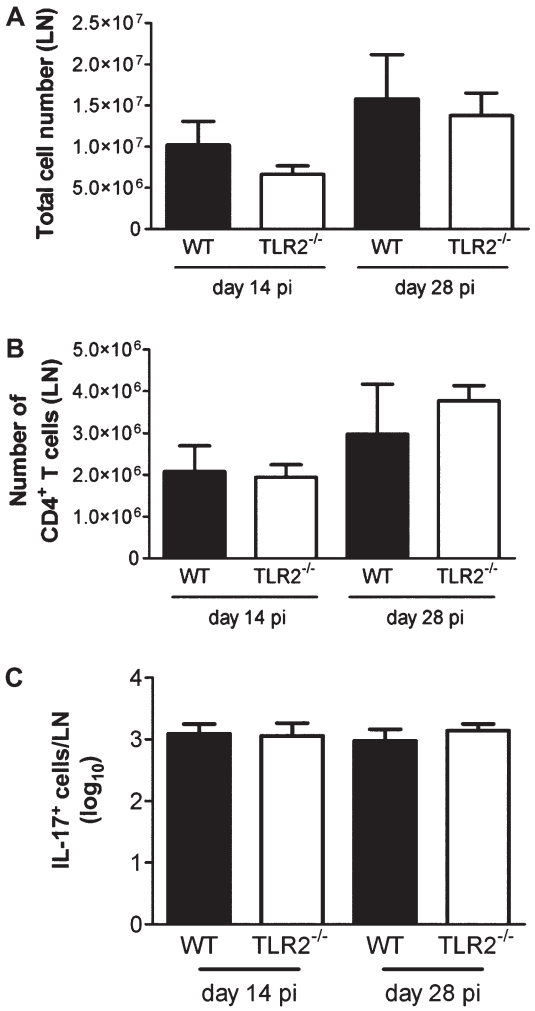

Fig. 3. Absence of TLR2 does not affect the number of T_h17 cells in the LN of *Mycobacterium tuberculosis*-infected animals. Wild-type or TLR2$^{-/-}$ mice were infected as in Fig. 1, and on days 14 and 28 post-infection, mediastinal LN were harvested, cell suspensions obtained and counted for total cell number determination (A) or stained with anti-CD4-antigen-presenting cell-specific antibodies for CD4$^+$ cell number determination by flow cytometry (B). Cells were also stimulated as indicated in Fig. 1 for the assessment of IL-17 production by ELISPOT (C). Data points show the mean ± SEM value for five to six mice per group and the significance was determined by the Student's *t*-test. The data are representative of two independent experiments.

TLR2 deficiency transiently delays lung inflammation in response to M. tuberculosis infection

Considering that both TLR2 and IL-17 have been implicated in the inflammatory response to *M. tuberculosis*, we questioned whether TLR2 deficiency impacts the kinetics of the lung inflammatory response following *M. tuberculosis* infection. We found that at day 54 post-infection, the lung inflammatory infiltration was reduced in TLR2$^{-/-}$ animals (Fig. 4A). However, at very late stages of infection, the histological pattern observed was similar, independently on the presence or absence of TLR2 (Fig. 4A). Despite the lower amounts of IL-17 and the significantly lower inflammatory index, in TLR2$^{-/-}$ mice, we did not observe a difference in the numbers of GR1$^+$ cells (likely neutrophils) in the lungs of TLR2$^{-/-}$ animals (Fig. 4B), as measured by flow cytometry. We observed that the number of iNOS-expressing foci (Fig. 4C), as assessed by immunofluorescence, was diminished in *M. tuberculosis*-infected TLR2$^{-/-}$ animals, 54 days post-infection. In line with the histological analysis, at late stages post-infection (215 days), both strains of animals showed similar iNOS expression (Fig. 4C). Our data thus show that TLR2 modulates the lung inflammatory response during *M. tuberculosis* infection.

Discussion

IL-17 has been recently reported as a key molecule for the development of mycobacteria granuloma (33) and lung pathology (41), in addition to its role on recall responses (40). In humans, altered T_h17 cells have been associated with either latency or more severe disease (42, 43). Thus, understanding the relative contribution of T_h17 cells in human *M. tuberculosis* infection and in animal models is of major interest. Therefore, a better knowledge of the molecular signals needed for *IL-17* expression, namely those involved in T_h17 cell establishment, during *M. tuberculosis* infections is important.

TLR2 has been positively implicated as a regulator of T_h17 cells in *Streptococcus pneumoniae* (50) or *Candida albicans* (51) infections, as well as in inflamed skin (52) and experimental autoimmune encephalomyelitis (53), indicating that TLR2 is a key molecule on the induction of IL-17-mediated immune response. However, in infections by *Staphylococcus aureus* (54) or by *Paracoccidioides brasiliensis* (55), TLR2 acts as a negative regulator of T_h17 cells. We now report that TLR2 signaling, although not required for the differentiation of *M. tuberculosis*-specific T_h17 cells in the draining LN of infected animals, is of extreme importance for the induction of p19 (and thus of IL-23) and maintenance of T_h17 cells at the site of infection. Our data are consistent with the well-known role for IL-23 in the maintenance of T_h17 cells (34,

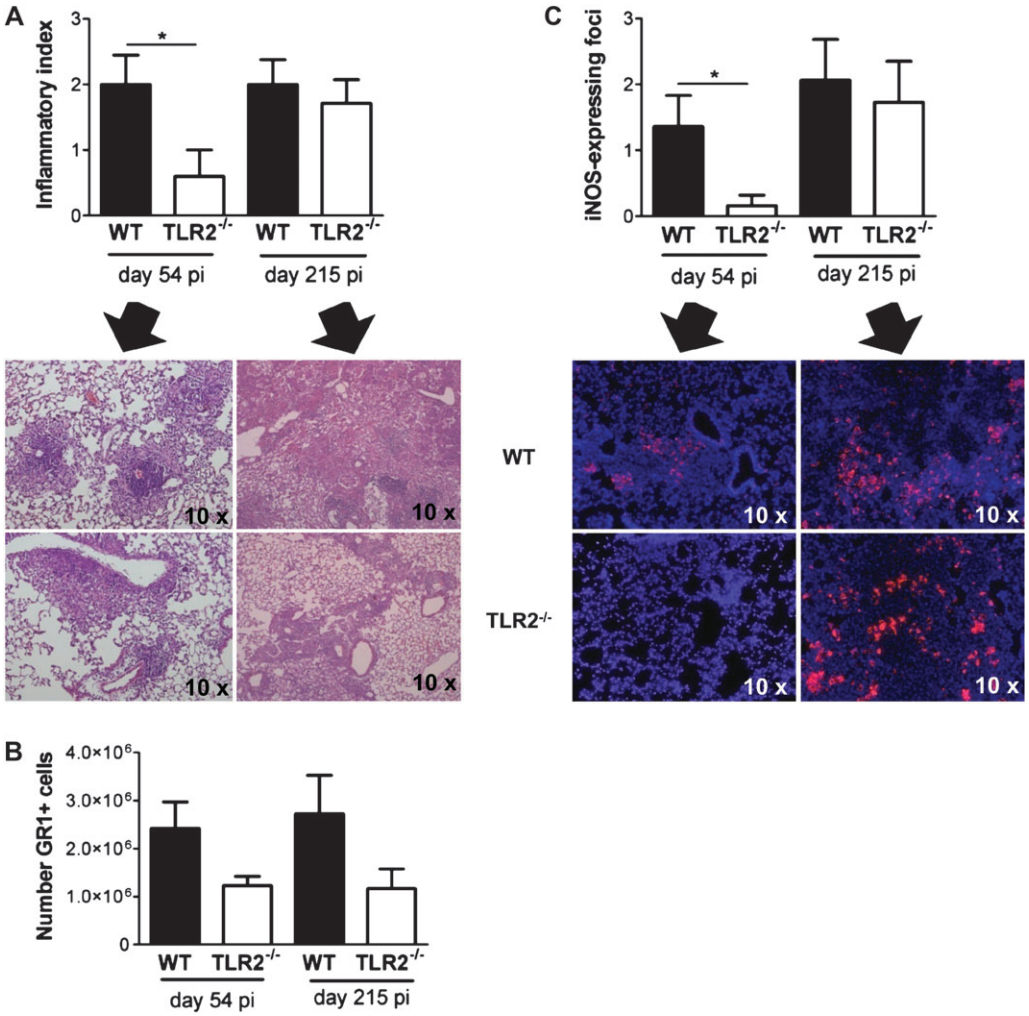

Fig. 4. TLR2 regulates lung inflammation during *Mycobacterium tuberculosis* infections. Wild-type or TLR2$^{-/-}$ mice were infected as before, and at the indicated time points post-infection, sections were prepared from formalin-fixed lungs. The degree of inflammation in the lungs of multiple mice was quantified in a blinded manner using a scale (the inflammatory indexes were: 0, absent; 1, mild; 2, abundant; 3, severe inflammation). The values from independent lungs were then combined to give a mean ± SD (A). At the indicated time points, lung cell suspensions were prepared and analyzed by flow cytometry for surface expression of GR1 (B). The number of iNOS-expressing foci in the lung tissue was determined by immunofluorescence, quantified and scored for each animal within the group (five to six animals). iNOS foci scores were no foci = 0; 0–10 = 1; 10–20 = 2; 20–30 = 3; >30 = 4 (C). Significance was determined by the Student's *t*-test (*$P < 0.05$). The data are representative of two independent experiments.

47, 56), and in line with a previous report showing that mice lacking p19 produce significantly lower levels of IL-17 in the lung during *M. tuberculosis* infection (39).

In addition to inducing IL-23, we suggest that *M. tuberculosis* recognition by TLR2 may also be involved in up-regulating the expression of other factors that mediate T_h17 cell responses. Consistently, in the absence of TLR2, we observed a decreased lung expression of IL-1β, another survival factor for T_h17 cells (56, 57). Furthermore, the expression of IL-6 in the lung was also decreased in TLR2$^{-/-}$-infected mice, which can contribute via defective IL-6-trans-signaling for defective T_h17 cell expansion and maintenance (58). In agreement with lower local T_h17 cell responses, we also found a decreased expression of CXCL9, CXCL10 and CXCL11 in the lungs of *M. tuberculosis*-infected TLR2$^{-/-}$ animals. These IL-17-induced chemokines were reported to participate in the recruitment, during recall responses, of CD4$^+$ T cells producing IFN-γ, which ultimately restrict bacterial growth (40). Taking into consideration that *M. tuberculosis*-specific T_h17 cells, following vaccination, are mainly resident in the lung and contribute to a faster recruitment of protective T_h1 cells (40), signals that sustain T_h17 responses, including as we now show those mediated by TLR2, may be of major interest to modulate the local immune response and potentiate vaccine efficacy.

Our study, placing TLR2 signaling as an important molecular mediator of effective T_h17 cell responses during *M. tuberculosis* infections, unveils a potential role for TLR2 signals during recall responses, such as during vaccination (a hypothesis that we are currently addressing) or re-infection. This is of particular importance as, in humans, polymorphisms in the *TLR2* gene have been associated to increased severity

of tuberculosis (19–24). Since most of the human studies have been performed in endemic areas, it is tempting to speculate that the problems faced by these individuals to control tuberculosis might be associated with impairment in mounting appropriate recall responses.

Previous studies suggested that mice lacking TLR2 suffered an exacerbated pathology in the lungs upon infection with *M. tuberculosis*, which was related to defective granuloma formation (10, 17, 18). In our study, we observed a transient delay in the lung inflammatory response of $TLR2^{-/-}$ mice, as assessed by histological analysis and quantification of iNOS-expressing foci. At late times post-infection, however, wild-type and $TLR2^{-/-}$ animals showed similar patterns of lung and liver (data not shown) inflammation, suggesting that the observed defect is transient and can be compensated overtime. It is conceivable that, in situations where $TLR2^{-/-}$ mice are unable to compensate the delayed inflammatory response in the lung, as it is the case of infection with high doses of inoculum (7, 18) or of combined TLR2/9 deficiencies (10), the control of bacterial growth may be put at risk.

Lung pathology during *M. tuberculosis* infection frequently associates with increased neutrophil influx and formation of pyogranuloma. $TLR2^{-/-}$ mice have been shown previously to have increased lung neutrophil influx (10, 17, 18). We did not observe a difference in the number of $GR1^+$ cells (likely neutrophils) in the lungs of *M. tuberculosis*-infected $TLR2^{-/-}$ animals. This discrepancy could be due to the fact that different routes and doses of infection were used. Of note, in a model of *M. avium* intravenous infection, $TLR2^{-/-}$ animals did not exhibit an enhanced influx of neutrophils into the liver (16).

Understanding the molecular signals that dictate T_h17 differentiation during infection gains a novel importance in light of the recent evidence for a role of IL-17 in determining protection versus pathology during infections by mycobacteria (33,41–43). Furthermore, considering the described role of T_h17 cells in accelerating T_h1 responses during secondary infections (40) and thus in increasing the efficacy of vaccination, TLR2-deficient signals, as our results now suggest, may compromise the efficiency of vaccination against tuberculosis and provide new targets for vaccine improvement.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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CHAPTER 4

DISCUSSION

Tuberculosis is a disease that still nowadays represents a problem of public health (1). In most cases, exposure to *M. tuberculosis* results in a chronic lung infection where the immune response is balanced enough to avoid major tissue damage as well as bacterial growth but is not efficient in clearing the pathogen (2, 3). Moreover, once this fine balance is broken, disease will progress. In 5-10% of cases, individuals are not able to establish chronic infection and develop active disease upon *M. tuberculosis* exposure (2, 3). Although *M. bovis* BCG has proven its efficacy in the protection against childhood tuberculosis (4, 5), the highly variable range of protection against adult tuberculosis is a serious problem (6-8). Therefore, to study the interplay between the host´s immune system and the infecting agent, *M. tuberculosis*, is of major interest.

In the literature, we find several reports that associate susceptibility to infection with impaired development, or absence, of responses dependent on molecules such as, IFN-γ, NOS2, αβ TCR, MHCII and TNF, leading to susceptibility features like increased bacterial burdens, histopathology (many times associated with disrupted granuloma formation) and decreased survival (9-11). However, the presence of the aforementioned players does not guarantee protection against infection as different strains of wild type mice show different levels of protection despite of all being able to develop protective pro-inflammatory immune responses (12). Interestingly, even in response to highly virulent mycobacterial strains, the levels of, for instance, IFN-γ are many times highly increased but with no benefit for the host (13, 14). It thus seems plausible that in order to be protected against infection the host needs to respond with the right threshold of pro-inflammatory responses. Importantly, an over-activated immune response could result in tissue damage so, to avoid this, the development of immune suppressive regulatory signals is also fundamental.

IL-10 has been described has an anti-inflammatory cytokine with a broad range of suppressive actions over the innate and adaptive immune responses and to be produced by cells from both arms of immunity. In the case of *M. tuberculosis* infections, human data has provided conflicting results regarding IL-10 polymorphisms and susceptibility to infection as it varies according to the region and ethnic background of the individuals (15). Despite this variation, IL-10 has been shown to be increased in patients with active disease (16-20), but whether this is meaningful for susceptibility or for protection against an over activation of the immune system, remains to be clarified and will probably depend on the threshold at which this cytokine is induced, the cell source and stage of infection. Although infection studies with IL-10 deficient

mice show variable results, culminating in late susceptibility, in protection or simply not influencing the course of infection (15, 21), there is data showing that when IL-10 is over expressed either by Th cells or macrophages, the host has a clear inability to control the mycobacterial infection (22-24), even though in some cases the levels of T cell IFN-γ responses remain intact (23, 24). Hence, it is possible that in situations when IL-10 production is increased, even if certain pro-inflammatory responses are in place, it might associate with a poor outcome for the host.

Alveolar macrophages and DCs are among the first cells to become in contact with mycobacteria once it is deposited in the lung, so the early recognition of the pathogen by these cells is very important to shape the immune response (25, 26). The recognition of mycobacteria by these cells occurs via several PRRs, namely TLR2, -4 and 9 (27-29) and culminates in the production of cytokines, such as IL-10, (27, 29) that will act as autocrine factors or, for instance, on adaptive immune cells (30). The role of these TLRs either in humans and in mouse models has produced disperse results as it seems to depend on the host genetic background, mycobacterial infecting strain and dose of infection (27). For that reason, more studies dissecting the contribution of all these factors and their co-relation with each other are necessary and of major importance.

In the case of macrophages, these effector innate cells characteristically stay in the lung upon mycobacterial infection initiating several microbicidal mechanisms. For this reason, the ability of macrophages to rapidly respond to mycobacteria is fundamental.

In chapter 3.1, we compared the regulation of IL-10 production by macrophages upon infection with two different strains of *M. tuberculosis* and found that preferential recognition of the bacterial strain either by TLR2 or TLR4 resulted in different IL-10 profiles. TLR2 signaling driven by *M. tuberculosis* H37Rv or Pam3Csk led to rapid and strong decrease of IL-10 mRNA stability whereas TLR4 signaling driven by *M. tuberculosis* Harlingen or LPS led to increased IL-10 mRNA stability, ultimately resulting in different secretion amounts by stimulated macrophages. Moreover, p38 has been identified has a key molecule to protect IL-10 mRNA from rapid degradation (31) in response to single TLR stimulation, however, in our study, for the first time, p38 activity is further associated with TLR4–induced TRIF activation, ultimately leading to increased amounts of IL-10 secretion by macrophages. We also found that this mechanism is recapitulated in response to infection with *E. coli*, possibly uncovering an IL-10 regulatory mechanism at the post-transcriptional level that might be conserved among pathogens that

strongly activate TLR4. It is thus fair to speculate that hosts infected with *M. tuberculosis* strains that primarily trigger TLR4 will be more prone to develop impaired protective responses, at least regarding the macrophage compartment, thereby compromising their ability to efficiently and rapidly initiate their microbicidal mechanisms. Having this in mind and i) reports showing the detrimental role of IL-10 overproduction during the course of *M. tuberculosis* (22-24), ii) increased IL-10 production associated with patients with active disease (16, 18, 19) and iii) increased IL-10 production in response to hyper-virulent strains when compared to less virulent strains (32, 33), our data highlights the possibility of TLR4–triggering acting as a virulence factor of *M. tuberculosis*, and possibly of other pathogens, via TRIF/p38-derived IL-10 mRNA regulation.

Considering the relevance of IL-10 in the innate immune response to mycobacteria, it is possible that, from the vaccination point of view, this cytokine might also play a detrimental role for the establishment of protective responses. In line with this, *Gopal et al* (34) showed that *M. bovis* BCG vaccination is more efficient in protection against *M. tuberculosis* infection when IL-10 is absent. Having these premises in mind, and considering the impact of DCs in initiating and shaping adaptive responses to mycobacterial infections, in chapter 3.2 we considered that to study the immune response triggered by *M. bovis* BCG in comparison to that of *M. tuberculosis*, is of importance for the design of new preventive strategies. In this study we found that, when compared to *M. tuberculosis*, *M. bovis* BCG was a stronger inducer of IL-10 and weaker inducer of IL-12 production by DCs. Considering these initial findings we questioned if *M. tuberculosis* and *M. bovis* BCG were triggering TLRs differently. In response to both mycobacteria TLR2 was important to drive IL-10 production and to lead to IL-12 inhibition. However, in the absence of TLR2, *M. tuberculosis* and *M. bovis* BCG induced similar amounts of IL-10 and IL-12, thereby suggesting that TLR2 has a major role in the regulation of the IL-10/IL-12 axis and that *M. bovis BCG* possibly triggers TLR2 in a stronger way that *M. tuberculosis* does. The fact that in the absence of TLR2 DCs are still able to produce IL-10 in response to infection suggest that other receptors might be involved in the production of this cytokine, as it is the case of dectin-1 that was shown to be able to induce IL-10 (35, 36). Moreover, previous reports have associated TLR-driven ERK activation with IL-10 induction and IL-12 inhibition (35, 37-39), which we also confirmed in our study as in the absence of ERK DCs have impaired IL-10 and increased IL-12 production in response to both mycobacteria. However, when ERK was inhibited, although *M. bovis* BCG still induced more IL-10 than *M. tuberculosis*, that difference was not as pronounced as when ERK was present indicating that, on one hand, ERK activation might be more

pronounced in response to *M. bovis* BCG and that other signaling molecules like p38 might account for differences induced by *M. bovis* BCG in respect to *M. tuberculosis*. In chapter 3.1 we found that differences in IL-10 production were due to TLR4/TRIF/p38 signals that protected IL-10 mRNA from rapid degradation *versus* TLR2/MyD88 signals that failed to promote the stability of the IL-10 mRNA. According to those results, it is possibly that the differences in IL-10 production by dendritic cells seen in chapter 3.2 might be, at least in part, also the result of post-transcriptional regulation differently induced by *M. bovis* BCG and *M. tuberculosis*. Whether the mechanisms operating for the stability of the IL-10 mRNA in DCs are the same as the ones in macrophages remains to be elucidated. However, since these two types of cells have distinct roles during infection it is possible that the mechanisms of IL-10 regulation will be different. Macrophages, which stay in the site of infection, need to respond rapidly regarding the initiation of microbicidal mechanisms and impairment of their effector activity by IL-10 over-production could be interpreted as a pathogen strategy to dampen the initial steps of the immune response. In the case of DCs, in particular regarding the chapter 3.2, these cells respond to *M. bovis* BCG with increased amounts of IL-10 and yet this strain is known to be less virulent than the *M. tuberculosis* H37Rv used in our study. It is possible that, by virtue of having several PRRs able to recognize mycobacteria, they could synergize for the production of IL-10 in the preferential recognition of *M. bovis* BCG in respect to *M. tuberculosis*. Regardless of variable IL-10 production by DCs as a virulence factor, it has become clear that *M. bovis* BCG vaccination in the absence of IL-10 results in increased protection (34) and is accompanied by increased production of IL-12 (chapter 3.2 and (34)) and increased Th1 type of responses (34). Therefore, identify and inhibit IL-10 derived molecules, or inhibit IL-10 itself, might prove of relevance to improve the protective immune responses (15, 40) such as the ones generated by *M. bovis* BCG (34).

In chapters 3.1 and 3.2 we explored innate immune recognition of mycobacteria via different receptors, namely TLR2, and found that this particular TLR is of major importance at defining IL-10 responses by macrophages and DCs. Several reports have shown that the *in vitro M. tuberculosis* recognition mediated by TLR2 on macrophages and DCs has also implications in cytokine response (41-43). Considering the role of TLR2 signaling in macrophages and DCs for cytokine production, it would be expected that mycobacterial recognition by TLR2 would impact protective immune responses. However, studies with TLR2 deficient mice show different results depending on the experimental set up. In response to low doses of *M. tuberculosis* inocula, although TLR2 deficient mice have similar bacterial burdens to wild type ones (41, 44-46), a

defective granuloma formation was observed (41, 44, 46, 47). Conversely, when high doses of inocula are used (44, 47) or TLR2 deficiency is combined with TLR9 deficiency (41), TLR2 shows to have a more pronounced role in protective responses against *M. tuberculosis*. The impact of TLR recognition in the development of protective Th1 type of responses has been a matter of interest but no definitive role has been established due to contradictory results (41, 45). Along with Th1 type of responses, also Th17 cells are generated upon mycobacterial infection. IL-17 has been identified has a major cytokine in the development of the granuloma (48), lung pathology (49) and is important to positively mediate protective immune recall responses (50). The recognition mechanisms and signals that lead to IL-17 production by γδ T cells and Th17 cells remain poorly understood (51, 52). Therefore, in chapter 3.3 we proposed to study the *in vivo* role of TLR2 at inducing and modulating the Th17 cells. We found that TLR2 absence did not have an impact in the generation of these cells in the DLNs, however, in the lung, there was a decreased expression of p19 (and thus IL-23) which was accompanied by a decreased number of Th17 cells. In agreement with lower numbers of Th17 cells, the chemokines CXCL9, CXCL10 and CXCL11 were also decreased in the lung. These IL-17-induced chemokines have been reported to participate in the recruitment, during recall responses, of IFN-γ producing Th1 cells responsible for the containment of bacterial growth (50). Considering the relevance of Th17 responses during vaccination (50), signals able to sustain Th17 responses are of major importance and we now show that TLR2 play an important role at generating those signals such as IL-23. Moreover, our study unveils a potential role of TLR2, via Th17 cells, during recall responses as it is the case during vaccination or re-infection. This is of great importance in humans were *TLR2* polymorphisms have been associated with severity of tuberculosis (53-57). Additionally, since most human studies come from individuals that live in endemic areas, it is possible that an impaired control of the disease might be associated with impaired recall responses.

Collectively, in the experimental work of this thesis, we focused on the impact that mycobacterial TLR recognition has on the regulation and production of IL-10 and on the maintenance of Th17 cells in the lung. This work allowed us to uncover a novel IL-10 regulatory mechanism in macrophages that is differently induced depending on TLR2 and TLR4 activation and revealed that *M. bovis* BCG is a potent IL-10 inducer on DCs, possibly compromising the generation of protective Th1 responses. Finally, we have attributed to TLR2 a new role as it

proved to be essential for the maintenance of Th17 cells in the lung upon *M. tuberculosis* infection, putting forward the possibility that TLR2 might be important for the generation of protective immune responses in the context of recall responses.

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