Universidade do Minho
Escola de Medicina

Pierre Kang Ngabe

The role of IL-23 in the immune response to *Mycobacterium tuberculosis*

O papel da IL-23 na resposta imunitária ao *Mycobacterium tuberculosis*

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Dissertação de Mestrado
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e do
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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

O papel da IL-23 na resposta imunitária ao *Mycobacterium tuberculosis*

O *Mycobacterium tuberculosis* é o agente etiológico da tuberculose e a principal causa de morte por um só agente infecioso em humanos. Esta bactéria é um exemplo ideal de um agente infecioso para o qual a resposta protetora depende da imunidade celular, uma vez que o organismo vive dentro de células, normalmente macrófagos. Assim, os mecanismos efetores mediados por células T, em vez de anticorpos, são necessários para controlar a infeção. No entanto, estas respostas demoram a ser ativadas sendo detetadas apenas 3 semanas após a infeção. Assim, a imunidade inata desempenha um papel crítico na proteção do hospedeiro contra a infeção com *M. tuberculosis*, visto que muitos indivíduos infetados controlam a infeção, apesar do atraso na resposta imunitária adquirida. Macrófagos infetados com *M. tuberculosis* produzem IL-23 em poucas horas de infeção; A nossa hipótese para este trabalho é que a IL-23 desempenha um papel fundamental na resposta inata contra *M. tuberculosis*, inclusive induzindo a expressão de peptídeos antimicrobianos que podem ajudar no controle da infeção. Por esse motivo, procuramos explorar o papel desta citoquina durante a infeção por *M. tuberculosis in vivo* e *in vitro*.

Começamos por determinar a expressão de peptídeos antimicrobianos por macrófagos infetados com *M. tuberculosis* na presença ou ausência de IL-23. Os nossos dados mostram que a IL-23 não induz a expressão de peptídeos antimicrobianos em macrófagos infetados por *M. tuberculosis*. De seguida, determinamos o papel da IL-23 na resposta imunitário e no controle do *M. tuberculosis* in vivo. Para isso, infetamos ratinhos deficientes em IL-23R e comparamos sua capacidade de controlar *M. tuberculosis* com a de ratinhos C57BL/6. Os nossos dados mostram que, durante as primeiras semanas de infeção a IL-23 pode desempenhar um papel fundamental no controle de *M. tuberculosis*. No entanto, durante os estadios finais da infeção, os ratinhos com deficiência no IL-23R são mais suscetíveis à infeção. Apesar disso, os nossos dados não revelaram alteração significativa na resposta imunitária protetora, no que diz respeito à ativação e função efetora de células T. Também determinamos o potencial da IL-23 na proteção conferida pela vacinação da mucosa. Mostramos que ratinhos vacinados com BCG e ratinhos vacinados com BCG+IL-23 controlam a infeção por *M. tuberculosis* da mesma forma; no entanto, ratinhos vacinados com BCG+IL-23 exibem uma arquitetura melhorada de tecido pulmonar, o que sugere um papel importante da IL-23 na vacinação via mucosa.

Em conjunto, os nossos dados justificam mais estudos para definir o papel da IL-23 durante a vacinação da mucosa e após a infeção por M*. tuberculosis*. Especificamente, o uso de uma dose maior de infeção poderá desvendar novos mecanismos inatos de proteção contra o *M. tuberculosis* mediados por IL-23.

Palavras chave: Citoquinas; Imunidade inata; Imunidade adquirida; Vacinação.

Abstract

The role of IL-23 in the immune response to *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is the etiological agent of tuberculosis and the leading cause of death due to a single infectious agent in humans. *M. tuberculosis* is an ideal example of a pathogen for which the protective response relies on cell-mediated immunity, since the organism lives within cells, typically macrophages. Therefore, T cell effector mechanisms, instead of antibody, are required to control the bacterium. However, these responses are slow to be activated and are detectable only 3 weeks after infection, giving the pathogen time to spread. Innate immunity plays a critical role in protecting the host from early infection with *M. tuberculosis*, as many *M. tuberculosis*-infected individuals can control the infection despite the delay in the acquired immune response. *M. tuberculosis*-infected macrophages produce IL-23 within hours infection; in this work, we hypothesized that IL-23 plays a pivotal role in the innate response to, including by inducing the production of antimicrobial peptides which may help in the control of infection. For this reason, we sought to explore the role of this cytokine during *M. tuberculosis* infection both in vivo and in vitro.

We began by determining the expression of antimicrobial peptides by *M. tuberculosis*-infected macrophages in the presence and/or absence of IL-23. We showed that IL-23 does not induce the expression of antimicrobial peptides. We next determined the role of IL-23 in the immune response and control of *M. tuberculosis in vivo*. To do this, we infected IL-23R-defficient mice and compared their ability to control *M. tuberculosis* with that of C57B/6 mice. We showed that during early infection IL-23 might play a key role in the control of infection. However, during the late stages of infection IL-23R-defficient mice were more susceptible to M*. tuberculosis* infection. Despite this, our data did not reveal significant alteration in the protective immune response, in what regard T cell activation and effector function. We also determined the potential of IL-23 in enhancing protection conferred by mucosal vaccination. We showed that mice vaccinated with BCG and mice vaccinated with BCG+IL-23 control *M. tuberculosis* infection equally; however, BCG+IL-23 vaccinated mice display improved lung tissue architecture suggesting a potential role of IL-23 in mucosal vaccination.

Taken together, these data warrant further studies to define the role of IL-23 during mucosal vaccination and following infection with *M. tuberculosis*. Specifically, the use of a higher dose of infection might unravel novel innate mechanisms of protection against *M. tuberculosis* mediated by IL-23.

Key words: Cytokines; Innate immunity; Acquired Immunity; Vaccination.

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LISTS OF ABBREVIATIONS

AFB, Acid-fast bacilli

AM, Alveolar macrophages

Ag, Antigen

AMPs, Antimicrobial peptides

APCs, Antigen-presenting cells

BCG, Bacillus Calmette-Guerin

BAL, Bronchoalveolar lavage

BMDM, Bone marrow derived macrophages

CD, Cluster of differentiation

cDNA, Complementary deoxyribonucleic acid

CO_2, Carbon dioxide

CFUs, Colony-forming units

CLR, C-type lectin receptor

CRAMP, Cathelicidin-related antimicrobial peptides

CTL, Cytotoxic T lymphocytes

CXCL, C-X-C motif chemokine ligand

DCs, Dendritic cells

DMEM, Dulbecco's Modified Eagle Medium

FACS, Fluorescence activated cells sorting

G-CSF, Granulocyte-colony-stimulating factor

GM-CSF, Granulocyte-macrophage colony-stimulating factor

iNOS, Inducible nitric oxide synthase

KO, Knock out

KLRG1, Killer cell lectin-like receptor subfamily G member 1

ILCs, Innate lymphoid cells

IL, Interleukin

IFN-γ, Interferon gamma

LTi, Lymphoid tissue inducer cells

MHC-I, Major Histocompatibility Complex class I

MHC-II, Major Histocompatibility Complex class II

MDR-TB, Multi-drug resistant tuberculosis

NOD-NLR, Nucleotide-binding oligomerization domain- like receptor

PD-1, Programmed cell death protein 1

PRRs, Pattern recognition receptors

RIG-I- RLR, retinoic acid-inducible gene I-like helicases receptor

RORγt, RAR-related orphan receptor gamma

ROS, Reactive oxygen species

STAT, Signal transducer and activator of transcription factors

TB, Tuberculosis

TCR, T-cell receptors

TGF-β, Tumour growth factor beta

Th, T- helper

TNF-α, Tumour necrosis factor alpha

TLR, Toll-like receptors

HIV, Human immunodeficiency virus

WHO, World health organisation

WT, Wild type

XDR-TB, Extensive drug resistant tuberculosis

Introduction

1- *Mycobacterium tuberculosis* incidence and prevalence

Mycobacterium tuberculosis (*M. tuberculosis*) is a nonmotile, nonsporulating, and slow growing bacterium (1,2,4, 5). Its defining feature is a waxy outer membrane, that is rich in mycolic acids, thus making it neither a Gram-positive nor Gram-negative acid-fast bacterium (AFB) (2-5). *M. tuberculosis* is part of a group of organisms classified as the *M. tuberculosis* complex, which include *Mycobacterium africanum, Mycobacterium canettii, Mycobacterium bovis,* and *Mycobacterium microti* (49). It belongs to the phylum Actinobacteria and is an obligate-aerobic, facultative intracellular major human pathogen, affecting mainly the lungs (2,4). Thus, making pulmonary disease the most common presentation. However, other organ systems that are commonly affected include the gastrointestinal system, the musculoskeletal system, the lymphoreticular system, the skin, the liver, and the reproductive system (49). *M. tuberculosis* has been suggested to have originated more than 150 million years ago, and an early progenitor of *M. tuberculosis* might have infected early hominids in East Africa approximately 3 million years ago. However, the common ancestor of modern strains of *M. tuberculosis* might have appeared around 20,000–15,000 BCE (6).

M. tuberculosis is the etiological agent of tuberculosis (TB) and the leading cause of death due to a single infectious agent in humans (2,5-7,21). Although the bacillus can infect other animals, however, evidence for zoonotic transmission to humans is scarce (21). Human TB has been suggested to have appeared about 70,000 years ago (7,21), with World Health Organization (WHO) estimating that around one fourth of the global population is latently infected with *M. tuberculosis*. About 5% to 10% of infected patients develop active TB, with reactivation higher in HIV co-infected patients. With 2017 statistics estimating that about 10 million people contracted TB and approximately 1.3 million related deaths (8,50), of which about 87% of patients are residing in developing countries and regions (50). Bacillus Calmette-Guérin (BCG), the attenuated strain of *Mycobacterium bovis* is the current vaccine against TB (6,9,). BCG has been in use since the 1920s, being efficient in infants and young children, preventing disseminated TB and tuberculous meningitis. However, BCG has shown variable and mostly poor protection against TB in adolescents and adults (9,10). Effective TB drugs are also available, which require an intensive treatment of at least 6 months daily to achieve a cure. However, there is global incidence of multi-drug resistant tuberculosis (MDR-TB), as well as more severe forms of resistance known as extensively drug resistant tuberculosis (XDR-TB) detected (4,5,8).

2- Innate Immunity to *M. tuberculosis* infection

Innate immunity plays a critical role in protecting the host from early infection with *M. tuberculosis*. This is highlighted by the fact that many *M. tuberculosis*-infected individuals being able to control the infection despite a delay of acquired immunity (100). Infection with *M. tuberculosis* occurs through the aerosol route. Lung resident myeloid cells such as alveolar macrophages (AMs) and dendritic cells (DCs) are the primary cells initiating first contact with the bacillus. AMs are specialized innate immune cells that reside in pulmonary alveoli and ingest the inhaled bacterium and are critical in setting the stage for the subsequent immune responses against *M. tuberculosis* (4,6,10). These cells recognise *M. tuberculosis* bacilli through expressing pattern recognition receptors (PRRs) such as Toll-like receptor (TLR), nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), C-type lectin receptor (CLR), and retinoic acid-inducible gene I (RIG-I)-like helicases receptor (RLR) (4,9-13,100).

Engagement of PRR with *M. tuberculosis* ligands induce actin polymerization (54) and subsequently, formation of phagosome. This is immediately followed by phagosome maturation. Phagosome maturation is characterised by fusion of the phagosome with early endosome, then late endosome, and finally with lysosomal compartments to form a phagolysosome. The phagolysosome is characterized by luminal acidification (pH of about 5), which alters its protein and enzymatic composition, for example, release of reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), autophagy, and apoptosis for *M. tuberculosis* clearance (50, 54).

However, *M. tuberculosis* can prevent phagosome maturation through inhibition of phagosome fusion with lysosomes (51,52), thus preventing phagosome acidification. This way, *M. tuberculosis* evades proteolytic degradation and late immunological events, such as antigen presentation required to initiate an adaptive immune response (52). Persistence of *M. tuberculosis* within vesicles is through association with Rab5 protein. Rab proteins control vesicular transport between organelles, with Rab5 and Rab7 serving as master regulators of transport along the endocytic and phagocytic pathways. Normally, early phagosomes recruit Rab5, which is subsequently replaced by late endocytic Rab7 during the maturation process that recruits downstream effectors to confer fusion competence with lysosomes (4,12).

M. tuberculosis uptake by AMs leads to secretion of various cytokines and chemokines that mediate systemic effects and recruit various inflammatory cells (54). The cytokine profile secreted by *M. tuberculosis*-infected AMs include pro-inflammatory cytokines, such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), IL-2, IL-6, IL-12, IL-18, IL-23, and granulocyte–macrophage colony-stimulating factor (GM-CSF), as well as anti-inflammatory cytokines including IL-27, IL-4, IL-10, IL-13, and tumour growth factor beta (TGF-β). AMs are crucial in maintaining airway immune

homeostasis due to their plasticity and polarization nature. In response to *M. tuberculosis* infection, pro-inflammatory cytokines including IFN-γ, TNF-α and GM-CSF can differentiate AMs into the classically activated M1 macrophages. These macrophages, M1, are key effectors of the host response against *M. tuberculosis* and promote Th_1 responses. M1 macrophages are necessary for effective bactericidal properties and effector responses that lead to the recruitment of various cell populations such as epithelioid cells, Langhans giant cells, mononuclear phagocytes, fibroblasts, and T and B lymphocytes to the site of the *M. tuberculosis* containing macrophages, thus promoting granuloma formation, that is the hallmark of TB (4,11,13). Contrarily, anti-inflammatory cytokines including IL-4, IL-13 as well as IL-10 and TGF-β can differentiate AMs into M2 macrophages, which are poor antigen-presenting cells and suppressors of Th_1 responses. M2 macrophages population have been shown to play important roles in maintaining the tight balance between exacerbated pathology and control of mycobacterial growth (4,11,13).

3- Adaptive immunity to *M. tuberculosis*

The containment versus spread of *M. tuberculosis* depends on the nature of the T-cell response that develops in response to infection (19). *M. tuberculosis* is an ideal example of a pathogen for which the protective response relies on cell mediated immunity. This is because the organism lives basically within cells, typically macrophages. Thus, T cell effector mechanisms, instead of antibody, are required to control or eliminate the bacterium (20). Effector Th_1 cells are differentiated from naïve T cell lymphocytes, that is driven by signals received from T-cell receptors (TCR), costimulatory receptors, and cytokines (21,55). *M. tuberculosis* residing within the vacuoles of infected DCs and macrophages, which are professional antigen-presenting cells (APCs) (10,20) and are key in linking innate and adaptive immunity through the secretion of IL-12 (56). This cytokine influences the migration APCs to the mediastinal lymph node, and after the presentation of *M. tuberculosis* antigens through the Major Histocompatibility Complex class II (MHC-II) molecule, activation, and differentiation of antigen specific $CD4^+$ T cells (55) by activating the signal transducer and activator of transcription (STAT) factors. STAT in turn induces the expression of T-bet, which is a master regulator of Th_1 cells. T-bet then binds directly to many Th_1-specific genes, resulting in differentiation and proliferation of T cells (21). The main function of effector Th_1 is the production of cytokines, particularly IFN-γ. This cytokine enhances macrophage microbicidal mechanisms through the activation of signalling pathways that include the iNOS pathway and induces the process of acidification and maturation of phagosomes and autophagy, that are responsible for the control of *M. tuberculosis*.

(21,57) Additionally, $CD4^+$ T cells can also produce other cytokines like TNF-α and IL-2 that act synergistically with IFN-γ to activate macrophages to control or eliminate *M. tuberculosis* (19,20).

On other hand, activated $CD8^+$ T cells also referred to as cytotoxic T lymphocytes (CTL) due to their potential to kill target cells, recognize *M. tuberculosis*-specific antigens as peptides presented through classical MHC-I molecules. The most important $CD8^+$ T cells cytolytic functions to kill *M. tuberculosis*-infected cells are through granule-mediated function such as perforin, which acts as a pore for delivery of other proteins into the target cell, and the production of granzymes and granulysin to induce apoptosis (22). $CD8^+$ T cells also produce IFN-γ and TNFα, cytokines that have critical functions during *M. tuberculosis* infection as discussed above (20,22); although, none of them can compensate for the absence of $CD4^+$ T cells. Studies in mice deficient in $CD4^+$ subset and Th_1 type cytokines, for example IL-12p40 and IFN-γ, have shown that these mice succumb early to *M. tuberculosis* infection with high bacterial loads. With similar effects observed in mice with the defects in enzymes involved in the generation of host-bactericidal molecules, dependent on IFN-γ axis (57).

However, the adaptive immune responses to *M. tuberculosis* are slow and are detectable about 3–8 weeks after infection, when $CD4^+$ T cells arrive in the infected lungs (10,19,20). This time lag between the establishment of infection and T cells arrival at infection site likely contribute to the inability of the host to clear the organism. A transient depletion of $CD11c^+$ cells has been shown to impair $CD4^+$ T-cell priming (10) due to reduction of MHC class II-antigen presentation by infected APCs (19). Additionally, studies on TB and HIV co-infection have provided important insights into adaptive T-cell responses to TB. Indeed, *M. tuberculosis* specific $CD4^+$ T cells are depleted from the periphery early after HIV infection in humans, suggesting there is a preferential targeting of *M. tuberculosis*-specific memory $CD4^+$ T cells by the virus. This reduction in $CD4^+$ T cells results in increasing reactivation of latent *M. tuberculosis* infection and altered histopathological characteristics of TB disease in HIV-infected patients, for example, diffuse necrotic lesions instead of structured granulomas. Consequently, these data support the concept that Th_1 are prerequisite for TB defence and act by stimulating the antimycobacterial immunity through the Th_1 to IFN-γ production, that activates macrophages for *M. tuberculosis* killing and restriction pathway (10,20,21,58).

4- IL-23 signalling and Immune responses in *M. tuberculosis* infection

Interleukin (IL-23) is a member of the IL-12 cytokine family (24-28). In both mice and humans (24), IL-23 is a disulphide-bonded heterodimeric cytokine composing of an IL-23-specific p19 subunit that is closely related in structure to IL-12p35, and a p40 or IL-12/IL-23p40 subunit common to IL-12 (27,29,30). IL-23 heterodimer signalling is through the IL-23 receptor (IL-23R), which is composed of a unique IL-23R and an IL-12Rβ1 subunits. IL-23R is predominantly found on activated memory T cells, NK cells, and innate lymphoid cells (ILCs), with lower levels of IL-23R on monocytes, macrophages and DCs (24-30). IL-23 has similar but not identical biological functions with IL-12, and both cytokines are secreted by human and mouse DCs and tissue-resident macrophages (24,26-29). DCs and macrophages are the central sensory components of the immune system, and they govern the balance between tolerance, ignorance, and immunity (29). These APCs secret the cytokine IL-23 in response to recognition of exogenous microbial components by Toll-like receptors or endogenous signals in favour of host defence and wound healing (24,29).

In addition to Th_{17}, ILCs or γδ T cells being the main sources of IL-17 are induced by IL-23 to produce IL-17 through RAR-related orphan receptor gamma (RORγt). In these populations, IL-23R activity can drive T-cell immune responses in the absence of T cell receptor engagement (24,25). During early *M. tuberculosis* infection, infected macrophages secret IL-23 in the lungs, that can occur within hours of pathogen encounter (25,27). The secreted IL-23 induces inflammatory effects on innate lymphoid cell 3 (ILC3) to produce IL-17 (24,26,30), which occurs as early as day 3 of infection (30). This early IL-17 production triggers neutrophil infiltration to the lung through induction of CXC chemokines such as IL-6, IL-8, and G-CSF (26,30,31). Innate IL-23 also exerts effects on myeloid cells to secret proinflammatory cytokines such as TNF and IL-1. IL-23 alone seems to be sufficient to induce IL-17 production from ILC3, although involvement of other innate receptors (such as TLRs) is not excluded (30). Additionally, IL-23 plays an important role in bridging innate and adaptive responses, which are less dependent on IFN-γ, in contrast to IL-12 (29). Unlike IL-12 that differentiates naïve T cells into Th_1 which are IFN-γ producing cells, IL-23 differentiates $CD4^+$ T cells into Th_{17} phenotype, resulting in the production of IL-17 (24,29,31) and can convert Tregs into Th_{17}-like cells to regulate the host immunity (24).

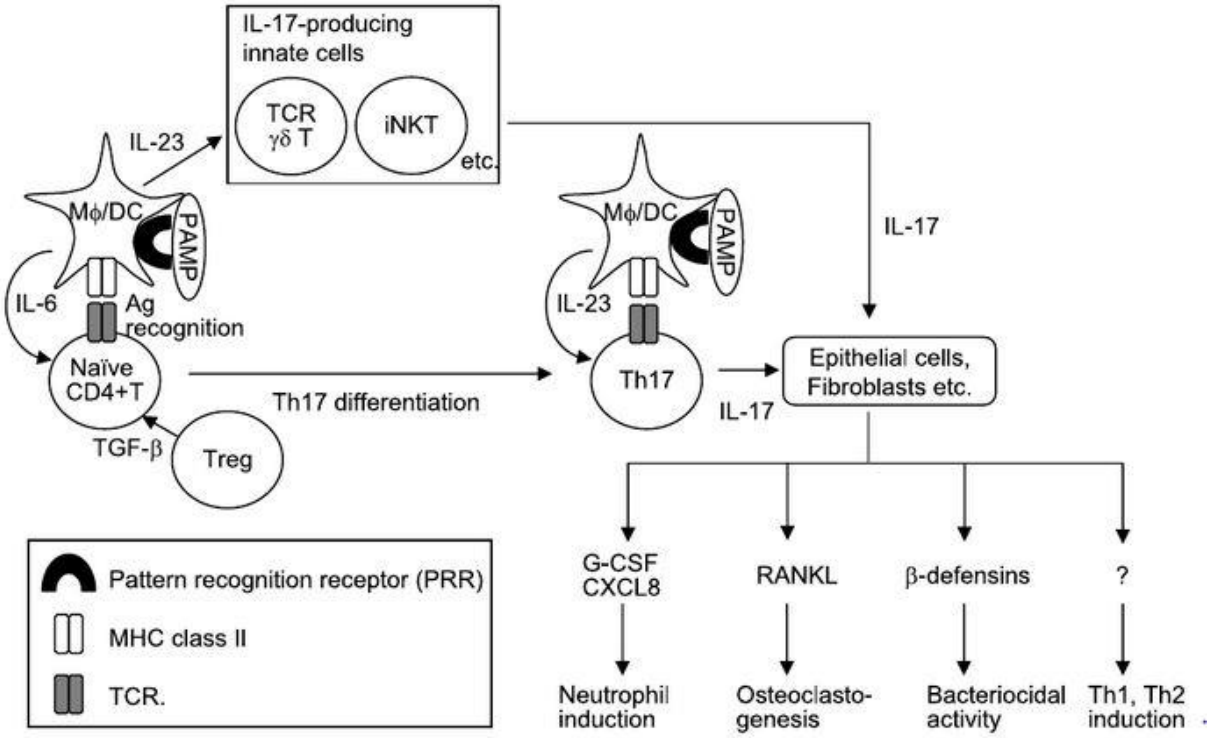

Figure 1. Regulation of IL-17 production and protective function of IL-17. IL-17 is not induced only in acquired immunity, but also in innate immunity and its functions are independent of its source. *Mycobacterium tuberculosis* pathogens induce IL-17 production by innate lymphocyte, through the production of IL-23. IL-17 produced in the innate immunity participates in protective response through killing of pathogens by neutrophils through their production of antimicrobial peptides. It also enhances antimicrobial peptides that are released by stimulated endothelial cells. Contrarily, differentiated Th_{17} can be stimulated to produce IL-17 in acquired immunity, that not only kill pathogens through neutrophils and antimicrobial peptides, but also enhances protective immunity through enhancement of Th_1 and induction of homing of effectorTh_1 cells into infected organs. Adapted from Matsuzaki, G. and Umemura, M., 2007.

5- IL-17 signalling and Immune responses in *M. tuberculosis* infection

Interleukin-17 (IL-17) is a multifaceted pro-inflammatory cytokine, with diverse roles both in immune-protection and immunopathology. It belongs to the interleukin-17 (IL-17) family consisting of six structurally related cytokines, which are IL-17A mostly referred to as IL-17, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. However, IL-17A and IL-17F are the most closely related, exist either as homodimers or as a heterodimer, and are usually co-produced. These cytokines, IL-17A and IL-17F signal through the

same heterodimer IL-17RA and IL-17RC receptor complex and largely share biological functions. IL-17 family signalling is through five members of the IL-17 receptors (IL-17R), consisting of IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE, and like their ligands, they share sequence homology (32-36). Of these receptors, IL-17RA is ubiquitously expressed on a wide range of tissues and cell types. IL-17A is the most extensively investigated cytokine of this family (33,35) and has a well-recognized role in immune surveillance at mucosa barrier surfaces (34). Upon signalling of IL-17 on the receptor IL-17RA, it initiates the activation of downstream signalling pathways to induce the production of pro-inflammatory molecules. Playing an essential role in host defence against microbial infections and implicating it in various inflammatory conditions such as autoimmune diseases, metabolic disorders, and cancer. IL-17A is a powerful inducer of inflammation due to its capacity to synergistically act with other cytokines like TNF-α. Where it helps in stabilizing the mRNA of the TNF-activated genes, which are inherently unstable, leading to amplification of TNF effects, to promote and prolong pro-inflammatory responses (33,35).

IL-17 is primarily secreted by T helper 17 (Th_{17}) cells that are differentiated from naïve T cells, under the influence of cytokines such as TGF-β, IL-6 and IL-1β. However, differentiated Th_{17} cells are stimulated to produce IL-17 by the cytokine IL-23, and it is also responsible for Th17 lymphocyte maintenance (36,38,39). IL-17 is also secreted by innate leukocytes including $\gamma\delta$ T cells such as ILC3s, lymphoid tissue inducer cells (LTi) and NK cells, as well as neutrophils and macrophages (34,36-38). Therefore, IL-17 production by innate cells may function as a bridge between innate and adaptive immunity and contribute to protective immunity against pathogens (39).

During early *M. tuberculosis* infection, macrophages and DCs secret IL-23, that is important in induction of the IL-17-producing cells. Although the Th_{17} response is effective in control of mycobacterial infections, the major IL-17-producing cells in the mycobacteria-infected lung are TCR γδ+ T cells (40,41). *M. tuberculosis*-infected DCs may also induce differentiated Th_{17} cells to release IL-17 by production of IL-23 (41). The most prominent role of IL-17 is to provide a protective inflammatory response towards pathogens at boundary tissues, such as lung. This occurs through inducing bronchial epithelial cells to release chemokines such as CXCL8 (36,42) CXCL1, CXCL2, CXCL5, CXCL6 that attract neutrophil to infection site and cytokines such as IL-6, G-CSF for granulocyte activation (36,42,43,45). IL-17 stimulation of bronchial epithelial cells enhances their expression of anti-mycobacterial peptides such the β-defensins (43,43) and mucins (MUC5AC and MUC5B) (45), that are key in immune resistance to mycobacterial infection.

Neutrophils on the other hand, are the first cells to infiltrate the lungs after *M. tuberculosis* infection. These cells are the most abundant cell type appearing in the bronchoalveolar lavage (BAL) in the sputum

of an active pulmonary TB patients (15). The accumulation and activation of neutrophil result in the production of antimicrobial peptides such as S100 proteins (S100A8, and S100A9) (13,36), cathelicidin (LL-37) and lipocalin 2 (11,44), as well as reactive oxygen species (ROS) for bacterial restriction. Apoptotic neutrophils and neutrophil granules, both of which contain active antimicrobial peptides can be phagocytosed by macrophages, leading to the inhibition of bacterial replication. However, factors released by neutrophils during respiratory bursts, such as elastase, collagenase, and myeloperoxidase, indiscriminately damage bacterial and host cells. Thus, neutrophils constitute a potent population of effector cells that can mediate both anti-mycobacterial activity and immunopathology during *M. tuberculosis* infection (11,13-15).

Additionally, it seems like the roles of $Th_{17/17}$ are dependent on the stage of infection, bacteria strains, or its burden. In the early developmental stage of initiating a protective immune response during *M. tuberculosis* infection, Th_{17} cells facilitate the recruitment of neutrophils, macrophages, and Th_1 cells to the area of inflammation and participate in the control of the infection process. These data show that Th_{17} responses are critical not only in the early activation of lung neutrophils but also in the development of Th_1 responses in *M. tuberculosis* infection. Indeed, studies have found that Th_{17} induces the expression of CXCl9, CXCL10, and CXCL11 chemokines, recruits IFNγ-producing cells, and thus ultimately restricts the reproduction of mycobacteria in macrophage in BCG vaccination model (65).

6- TB granuloma

Granuloma is the hallmark of TB, that is key in controlling and maintaining infection. Granulomas are characterised by a central acellular necrotic core, the caseum, arising at M. tuberculosis infection sites to create locations where there is active interchange between the host and the pathogen. Granuloma is formed through an early recruitment and clustering events involving inflammatory macrophages, which results in further recruitment of new macrophages and other immune cells such as dendritic cells, and multinucleate Langerhans and giant cells to the site of infection. Variable amounts of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes, and plasma cells are also recruited around these structures to create a wall (46-48). Importantly, granulomas mainly contain macrophages that are at various stages of activation, and T cells and B cells, but they can also contain neutrophils, dendritic cells, and fibroblasts (47,48). During progressive disease, the lesion enlargement and progressive necrosis results in erosion of vascular and bronchial walls, allowing spillage of infectious pathogens into the airways, thereby promoting disease transmission. However, in a restrictive granuloma, cells become more organized, and the lymphocytic rim is reinforced by the presence of organized fibrotic tissue, leading to containment of necrosis,

reorganization of cellular infiltrates, resolution of pathology prevents spread of the infection. T-cell defences are key for restrictive granuloma development, since mice lacking $CD4^+$ T lymphocytes have shown rapid progressive TB characterized by massive infiltrates of neutrophils and macrophages, leading to fulminant inflammation, necrosis, and bacterial dissemination to numerous organ systems (48). Increasing TB pathology have been associated with HIV and *M. tuberculosis* co-infection. This is caused by a functional disruption of the local immune response within the granuloma. This disruption is because of an increase in the viral load within involved tissue, leading to a decrease in the total number of $CD4^+$ T cells, along with a perturbation of *M. tuberculosis*-specific T cell function that leads to functional and detrimental changes within granulomas (59).

Objectives

The control or elimination of *M. tuberculosis* infection relies mainly on the adaptive immune responses. However, these responses are slow and are detectable 3 weeks after infection, giving the pathogen time to spread. Therefore, we sought to explore the role of the innate cytokine IL-23. IL-23 is produced by infected macrophages within hours of *M. tuberculosis* infection and plays a pivotal role in inducing immune cells to produce antimicrobial peptides which may help control *M. tuberculosis* growth. Thus, to better understand of the role of IL-23, we carried out the following aims:

1. To characterise the expression of specific antimicrobial peptides by *M. tuberculosis* infected macrophages in the presence and/or absence of IL-23.
2. To define the role of IL-23 in the immune response and protection against *M. tuberculosis.*
3. To test the potential of IL-23 in enhancing protection against *M. tuberculosis* induced by mucosal vaccination.

Materials and methods

1- Mice and infection

Mice used in this study were bred in the Life and Health Research Institute (IVCS) animal housing facilities. C57BL/6 were originally obtained from stocks purchase form Charles River laboratory (Barcelona, Spain) whereas, Interleukin 23-receptor deficient (IL-23R KO) mice were obtained from Andrea M. Cooper Laboratory (University of Leicester, Leicester, UK). Both male and female mice between the ages of 6 and 12 weeks old were used for experimental procedures.

Mice were infected through aerosol route with 280 CFU of *M. tuberculosis* H37Rv originally obtained from the Trudeau Institute (Saranac Lake, NY). At selected time points after challenge, mice were killed by CO_2 asphyxiation and the organs were aseptically excised and individually homogenized in saline. Organ homogenates were then 10-fold serial diluted and plated on nutrient 7H11 agar (BD Biosciences) for 3 weeks at 37°C, at which point CFUs were counted.

All procedures involving live animals were performed in accordance with the European Directive 86/609/EEC and approved by the *Subcomissão de Ética para as Ciências da Vida e da Saúde* (SECVS 074/2016) and the Portuguese National Authority *Direcção Geral de Alimentação e Veterinária* (DGAV 014072).

2- Bone marrow derived macrophages (BMDM), infection and/or IL-23 stimulation

C57BL/6 mice were euthanised through CO_2 suffocation. Incisions were aseptically made at the abdomens to remove the skin from the abdomen, including the hind limbs which were then cut and removed at joints and cleaned. The extremities of femurs and tibias were cut using scalpels, and marrow flushed out with supplemented DMEM, using a 2.5 ml syringe with 26G needle. Cells were then strained with 70 µm nylon strainer and centrifuged at 1200rpm for 10 minutes at 4°C. After centrifugation, pellets were resuspended and lysed with lysis buffer containing (1.6M NH_4Cl, distilled H_2O and 1XPBS) for 2 minutes. After 2 minutes, the lysis reaction is stopped by adding supplemented DMEM, centrifuged and pellets resuspended again in supplemented DMEM. Cells were counted and plated in 24 well plates at a density of 5x10^5 cell/ml with 0.02 µg/ml of macrophage colony stimulating factor (M-CSF), to differentiate cells into macrophages (64) and incubated for 4 days at 37°C in 5% CO_2 atmosphere. Cells morphology was monitored microscopically for differentiation and attachment to the bottom of wells. Cells were further stimulated on day 4 with 0.02 µg/ml M-CSF and incubated for 3 additional days at 37°C, 5% CO_2. At day 7, stock H37Rv strain of *M. tuberculosis* cells were taken from -80°C and dethawed in the safety cabinet. The bacteria cells were resuspended in the vial to remove clumps using a 1ml syringe with

a 26G needle through the tight-fitted vial cap. Cells were then infected with 2.5×10^5 bacteria/well and stimulated with 20ng/ml of IL-23.

3- RNA extraction and quantification

For RNA extraction, cells were lysed with 300 µl TRIzol (Life technologies) at different time-points and store at -80◦C. Lysed cells were thawed, scraped, and transferred into eppendorf tubes. 1 µL of RNase-free glycogen (20µg/µL) (Roche Diagnostics) per 300 µl TRIzol was added to increase recovery of nucleic acid. This was followed by the addition of 30µl Chloroform (CARLO ERBA Reagents), per 300 µl TRIzol, to promote phase separation and isolation of RNA from DNA and proteins. Tubes were then securely capped, vortexed vigorously for 15 seconds, and centrifuged for 15 minutes at 13000 rpm at, 4◦C. After centrifugation, the upper colourless aqueous phase was recovered and transferred into new Eppendorf tubes. An equal volume of Isopropanol was added to the eppendorf tubes for RNA precipitation. Samples were then incubated overnight at -20◦C. After overnight incubation, samples were centrifuged at 13000 rpm for 15 minutes at 4◦C and the supernatants were carefully discarded. RNA pellets were washed twice by resuspending in 800µL of 70% ethanol, vortexed and centrifuged at 9000 rpm for 5 minutes at 4◦C. The supernatant was discarded, RNA pellets dried and solubilized by resuspending in 11.5µL RNase/DNase-free water. RNA concentrations were measured by Nanodrop ND-1000 Spectrophotometer and the purity assessed through the A_{260}/A_{280} and A_{260}/A_{230} ratios

4- Reverse transcription (cDNA synthesis)

The synthesis of cDNA was carried out by reverse transcribing the extracted RNA using the GRS cDNA synthesis Master Mix (Grisp) according to the manufacturer's instructions. The volumes of RNA added to each reaction mix was based upon the concentrations of each sample. The volumes of RNA and RNase free water together was 9µl, and 10µL of (2X) Mastermix (containing a balanced concentration of oligo(dT), random hexamer primers, dNTPs, and RNase inhibitor), making a total volume of 19µl. The reaction mix were then heated in the thermocycler or thermoblock for 5 minutes at 65◦C to remove possible RNA structures and placed on ice for 2 minutes. 1 µL Xpert RTase (200U/µL) was then added to the reaction mixtures. Samples were thoroughly mixed and briefly centrifuged. After brief centrifugation, the mixtures were incubated in the thermocycler at 50◦C for 15 minutes for the reverse transcription reaction process. Reaction enzymes were later inactivated by heating at 85◦C for 5 minutes. The resultant cDNA templates were used to quantify the expression of target genes by quantitative real-time polymerase-chain reaction (RT-PCR).

5- Quantitative real-time polymerase-chain reaction (RT-PCR)

For SYBR green reactions, 1μL of cDNA sample was mixed with 9μL of reaction mix containing 2.8μL of RNA-se free water, 1μL of 10μM forward and reverse specific primers each, 0.2μl of ROX and 5μL of SYBR green qPCR Master Mix (KAPA) containing DNA polymerase, MgCl2, the fluorescent dye, SYBR Green. RT-PCR was performed in a CF*96TM Real-time system (Bio-Rad) using the following program: 95◦C for 15 min, followed by 40 amplification cycles at 95◦C for 15 seconds and 60◦C (or less, depending on the primer) for 30 seconds. Relative mRNA expression of the target gene was normalized to the levels of the housekeeping gene ubiquitin using the ΔCt method as follows: 1.8^(Housekeeping gene mRNA expression – Target gene mRNA expression) x 100000.

5.1- Primers (IDT-Integrated DNA Technologies)

Ubiquitin: (housekeeping gene); Fw, 5`- GGC TAT TAA TTA TTC GGT CTG CAT-3' Rv, 5'- GCA AGT GGC TAG AGT GCA GAG TAA -3', Tm (60◦C).

Cytokines:

TNF-α; Fw, 5'-GCC ACC ACG CTC TTC TGT CT-3´ Rv, 5'- TGA GGG TCT GGG CCA TAG ACC-3', Tm (60◦C), **IFN-γ;** Fw, 5'- CGG CAC AGT CAT TGA AAG CC- 3' Rv, 5'- TGT CAC CAT CCT TTT GCC AGT-3', Tm (62◦C), **IL-6;** Fw, 5'- ACA CAT GTT CTC TGG GAA ATC GT-3' Rv, 5' - AAG TGC ATC ATC GTT GTT CAT ACA-3', Tm (62◦C), **IL-10** Fw, 5'- ATT TGA ATT CCC TGG GTG AGA AG-3' Rv, 5'- CAC AGG GGA GAA ATC GAT GAC A-3', Tm (60◦C), **IL-17A;** Fw, 5'- ATC CCT CAA AGC TCA GCG TGTC-3' Rv, 5'- GGG TCT TCA TTG CGG TGG AGAG-3' Tm (60◦C), **IL-17F;** Fw, 5'- CTG TTG ATG TTG GGA CTT GCC-3' Rv, 5'- TCA CAG TGT TAT CCT CCA GG-3', Tm (60◦C(56◦)), **p19(IL-23);** Fw, 5'- CGT ATC CAG TGT GAA GAT GGT TGT-3' Rv, 5'- GCT CCC CTT TGA AGA TGT CAGA-3', Tm (60◦C), **p40(IL-12);** Fw, 5' - GCA GCG TGG GAG TGG GAT GTG - 3' Rv, 5'- GGG CAT CGG GAG TCC AGT CCA -3, Tm (60◦C), **IL-17A;** Fw, 5'**-** ATC CCT CAA AGC TCA GCG TGTC- 3' Rv, 5' -GGG TCT TCA TTG CGG TGG AGAG- 3', Tm (60◦), **IL-17F;** Fw, 5' -CTG TTG ATG TTG GGA CTT GCC- 3' Rv, 5' -TCA CAG TGT TAT CCT CCA GG- 3', Tm(60◦(56◦)).

2.4.3- Antimicrobial peptides (AMPs)

S100A9: Fw, 5'- CCC TGA CAC CCT GAG CAA GAA G-3' Rv, 5'-TTT CCC AGA ACA AAG GCC ATT GAG -3', Tm (60◦C), **S100A9-1;** Fw, 5'- CAC AGT TGG CAA CCT TTA TG- 3' Rv, 5'- CAG CTG ATT GTC CTG GTT TG-3', Tm (60◦C), **S100A8**: Fw, 5'- CCC GTC TTC AAG ACA TCG TTT G- 3' Rv, 5'- ATA TCC AGG GAC CCA GCC CTA G-3', Tm (60◦C), **S100A8-1**; Fw, 5'- GGC CAG AAG CTC TGC TAC TC-3' Rv, 5'-TGC

GAT GGT GAT AAA AGT GG-3', Tm (60°C), **Lipocalin-2**; Fw, 5'- TGC CAC TCC ATC TTT CCT GTT- 3' Rv, 5'-GGG AGT GCT GGC CAA ATA AG-3', Tm (60°C), **CRAMP (LL-37);** Fw, 5'- GCC GCT GAT TCT TTT GAC AT- 3' Rv, 5'- ATT CTT CTC CCC ACC TTT GC- 3', Tm (60°C).

6- Preparation of bronchial alveolar lavage (BAL) cell suspension for FACS

Mice were euthanised through CO_2 suffocation. Thorax and neck regions were aseptically opened to expose the lungs and trachea. To collect BAL fluid, a 21-gauge needle was inserted into the trachea and fastened with a string to avoid needle displacement. Using a 2.5 ml syringe, chilled phosphate buffer saline (PBS) was flushed into the lungs and withdrawn. This process was repeated several times to obtain a volume of about 4ml of BAL fluid. BAL samples were centrifuged at 1200 rpm for 8 minutes at 4°C and supernatants retrieved. Pellets were resuspended and red blood cells lysed with lysis buffer containing (PBS, distilled water (H_2O) and 1.6M NH_4Cl) for 1 minute while shaking. After 1 minute, DMEM (gibco) supplemented with (10% FBS, 1% antibiotics, 1% glutamine, 1M HEPES buffer and 100mM Sodium pyruvate), was added to stop lysis reaction. Samples were then centrifuged at 1200 rpm for 8 minutes at 4°C. Supernatants were discarded and pelleted cells resuspended with supplemented DMEM without antibiotics.

7- Preparation of lung parenchyma for colony forming units (CFUs)

For preparing lungs for CFU, Lung tissues were aseptically excised and put in Glass tubes containing sterile PBS. Lungs were then homogenized to release *M. tuberculosis* within tissues and granulomas, resulting in the formation of homogeneous solutions. Serial dilutions were performed from each sample, pipetting 100 µl of solution into eppendorf tubes containing 900 µl of sterile PBS. Each dilution step was preceded by inoculation of 7H11 agar plates with four quadrants that were previously treated with 70 µl PANTA antibiotics. After inoculation, agar plates were incubated at 37°C with 5% CO_2. CFUs were counted 3 weeks after incubation.

8- Preparation of lung single cell suspensions for flow cytometry

Lung tissues were excised and put into DMEM (gibco). Tissues were then chopped into tiny pieces in a petri dish using scalpels and transferred into 15 ml tubes to be incubated with collagenase to break peptide bonds within tissues, for 30 minutes, at 37°C with 5% CO_2. After incubation, tissues were macerated in a 70 µm nylon cell strainer placed in a petri dish with supplemented DMEM (gibco). Single cells were later transferred into 15 ml tubes and centrifuged at 1200 rpm for 8 minutes at 4°C.

Supernatants were retrieved and pelleted cells were resuspended, and red blood cells lysed with lysis buffer (PBS, distilled H_2O and 1.6M NH_4Cl), for 2 minutes while shaking. Lysis reaction was stopped by adding supplemented DMEM into tubes and centrifuged for 8 minutes at 1200 rpm, supernatants discarded, and pellets resuspended. For gradient separation of cells, 40% Percoll was pipetted into suspended cells, followed by a slow addition of 80% Percoll below the 40% percoll. Cells were then centrifuged at 1600 rpm for 32 minutes, at room temperature. After centrifugation, rings of cells were recovered using Pasteur pipettes and transferred into tubes containing supplemented DMEM. Cells were then centrifuged at 1200 rpm for 10 minutes at 4°C, and supernatants were discarded, pellets resuspended with supplemented DMEM without antibiotics.

9- Fluorescence activated cells sorting (FACS) staining

For surface antigen staining, cell suspensions from both BAL and lung tissues were plated in 96 well plates. Both BAL and lung parenchyma samples were stained for myeloid (60) and lymphoid cell (61) populations. Before staining, cell suspensions in plates were washed twice with FACS buffer (PBS, 10mM $NaNO_3$ and 10% FBS), centrifuged at 1500rpm for 5 minutes at 4°C. Surface antigens were then stained with 25µl/well of antibody cocktail. For lymphoid cells surface staining, the antibody cocktail added contained the following antibodies: CD45 (BV510, BioLegend), CD4 (APC/C47, BioLegend), CD8 (BV711, BioLegend), CD3 (BV605, BioLegend), CD19 (BV650, BioLegend), KLR61 (APC, BioLegend), CD69 (PE, BioLegend), CD44 (PerCp/Cy5.5, BioLegend) and PD (PE-e/Cy7, BioLegend). For myeloid cells surface staining, antibody cocktail contained the following antibodies: MHC II (FITC, BioLegend), Singlec-F (PE, BioLegend), Ly6C (PerCp/Cy5.5, BioLegend), CD11b (PE-Cy7, BioLegend), CD103 (APC, BioLegend), CD24 (BV510, BioLegend), CD45 (BV510, BioLegend), CD11c (BV605, BioLegend), CD64 (BV711, BioLegend) and Ly6G (BV785, BioLegend). Cells were incubated for 30 minutes at 4°C in the dark, and then washed with FACS buffer. After washing, cells were fixed with 2% PFA for 1 hour at 4°C, washed, resuspended in FACS buffer, and analysed for flow cytometry.

Samples were acquired using an LSR-II flow cytometer (BD Biosciences). For myeloid cells, analysis was as follows: Alveolar Macrophages ($CD45^+CD11c^+CD64^+F4/80^+Siglec\ F^+$) and Neutrophils ($CD45^+CD11b^+Ly6G^+$) (62). For lymphoid cells, analysis was as follows, $CD4^+$ T cells ($CD45^+CD3^+CD4^+$) and $CD8^+$ T cells ($CD45^+CD3^+CD8^+$) (63) early activation marker ($CD45^+CD69^+$) and activation marker ($CD45^+CD44^+$) (72), ($CD45^+PD\text{-}1^+$) (73) and ($CD45^+KLRG1^+$) (74). The percentages of cells were determined using the BD FACSDiva software and the analysis using Flow Jo software.

10- Vaccination and Histology

For vaccination, mice were vaccinated intranasally with 10^6 CFUs of BCG and rested for 60 days before aerosol infection with *M. tuberculosis*.

The upper-right lungs were antiseptically cut, inflated with 4% PFA and submerged in the same solution for 48 hours. For histological analysis, these sections were embedded in paraffin and sectioned into 2-3 µm thickness slices and then stained with haematoxylin and eosin.

11- Statistical analysis

Data were analysed using GraphPad Prism 7.01. Differences between the infected and uninfected was analysed using a 2way ANOVA with multiple comparisons. The results were given as means ± standard error (SE). Results were considered significant for $p \leq 0.05$. The following p-values were considered: *p= 0.0122; **p= 0.0095; ***p= 0.0003; ****p< 0.0001.

Results

Chapter I: Characterising the expression of specific antimicrobial peptides by *M. tuberculosis* infected macrophages in the presence and/or absence of IL-23.

1- Macrophage cytokines response to *M. tuberculosis* infection

M. tuberculosis and its protein and non-protein antigens are strong stimuli for the induction of cytokines in phagocytes (66). Indeed, macrophages are key innate immune cells against *M. tuberculosis* infection through production of cytokines (71). Macrophages can protect tissue integrity and heal damaged tissues, or under different contexts, can be major destroyers of tissues, due to their production of inflammatory cytokines and proteolytic enzymes. This is mostly due to different transcriptional programmes that are specifically activated by locally expressed signals (67).

Since heterogeneity and plasticity are hallmarks of mononuclear phagocytes and are not like any other cell type, we sought to analyse the various cytokines produced by macrophages following *M. tuberculosis* infection.

To establish the cytokine profile in the early stages of macrophages infection, we differentiated Bone Marrow Derived Macrophages (BMDM) from uninfected mice, infected them with *M. tuberculosis,* and at 3, 6 and 24 hours post-infection, performed gene expression analysis by RT-PCR. We found that the proinflammatory cytokines IL-1β, IL-6 and p19, a component of IL-23 was increasingly expressed over time (figure 2A, 2B and 2C). The expression of TNF-α increased during the first 6h of infection followed by a significantly drop in expression by 24h post-infection (figure 2D). On the other hand, we found that there was significance increase in the expression of p40, IL-17A and IL-17F between *M. tuberculosis*-infected and uninfected BMDM (figure 2E, 2F and 2G).

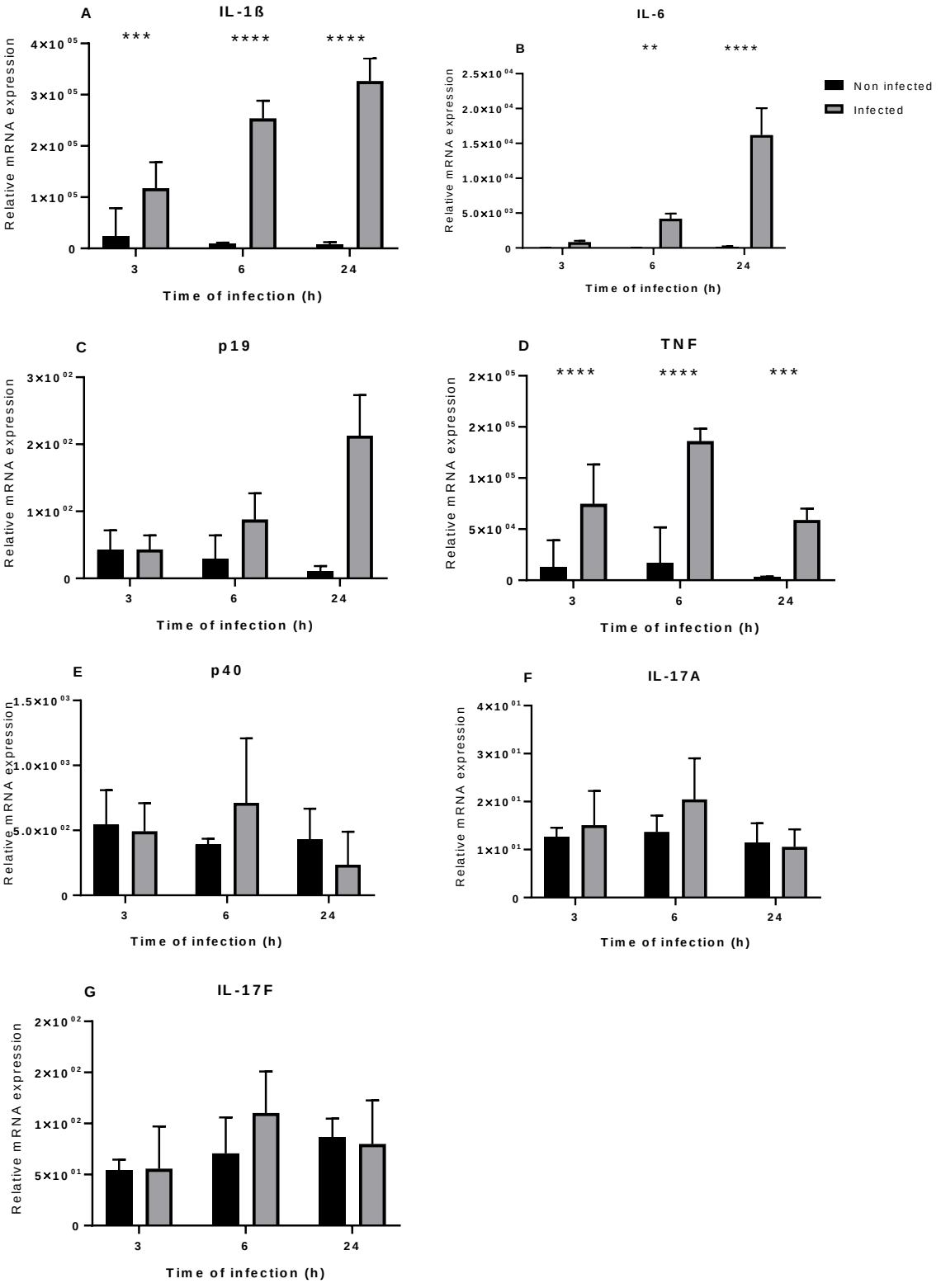

Figure 2. Early expression of proinflammatory cytokines by *M. tuberculosis* infected macrophages. Representative RT-PCR gene expression analysis of proinflammatory cytokines by *M. tuberculosis* infected macrophages at 3h, 6h and 24h post-infection. Figure **A, B** and **C** showing increasing accumulation of IL-1β, IL-6 and p19 post-infection. Figure **D** showing decreasing expression of TNF-α after 6h post-infection. Data

representatives were analysed by 2way ANOVA with p-value, ****, p< 0,0001; ***, p= 0,0015. Figure **E, F and G** showing no statistical significance in expression of p40, IL-17A and IL-17F post-infection.

We then analysed the expression of anti-inflammatory cytokines that have been described to inhibit the mycobactericidal activity of macrophages. We found increased expression of the anti-inflammatory cytokine IL-10, which was highest at 3h of infection, declining thereafter. These data were different from the expression of the anti-inflammatory cytokine TGF-β, as our data show that uninfected cells expressed higher amounts of TGF-β throughout infection (figure 3A and 3B).

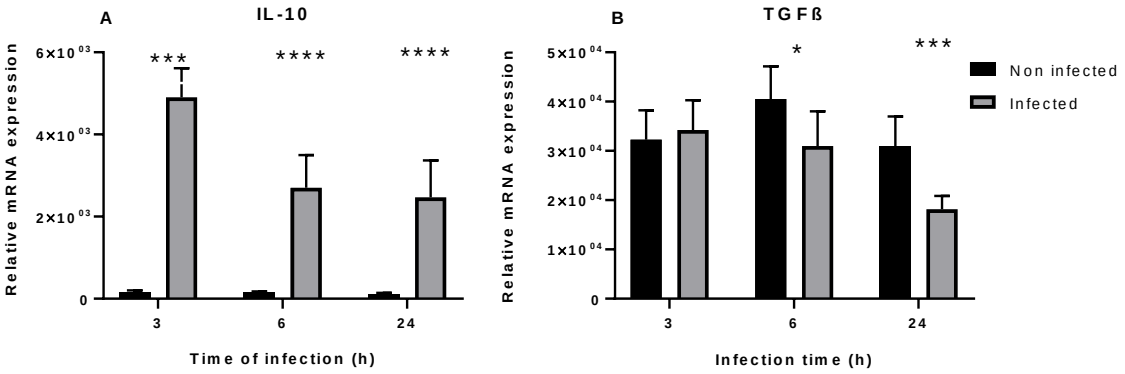

Figure 3. Early expression of anti-inflammatory cytokines by *M. tuberculosis*-infected macrophages. Representative RT-PCR gene expression analysis of anti-inflammatory cytokines by *M. tuberculosis* infected macrophages at 3h, 6h and 24h post-infection, figure **E** showing increasing expressions of IL-10 early infection. While figure **F** showing higher expression of TGF-β in uninfected cells post-infection. Data representatives were analysed by 2way ANOVA with p-value, **** p< 0,0001 and * p= 0,0133.

The above data above show that *M. tuberculosis*-infected macrophages produce inflammatory cytokines early during infection. While we observed that IL-6, IL-1β, IL-23 were increasingly expressed, the major innate cytokine TNF-α decreased in expression following infection. These observations highlight the importance of the balance in inflammatory cytokines created through IL-10 and TGF-β expression.
With these data showing the expression of IL-23 early following infection, we wanted to determine the role of this cytokine in the expression of antimicrobial peptides by *M. tuberculosis*-infected macrophages.

2- Expression of antimicrobial peptides by *M. tuberculosis*-infected macrophages.

As described above, our data show that *M. tuberculosis*-infected macrophages expressed inflammatory cytokines early during infection. We reasoned that the innate cytokine IL-23 can be inducing *M. tuberculosis*-infected macrophages to produce antimicrobial peptides, for example Lipocalin-2 (68), LL-37/Cathelicidin (69) and Calprotectins(S100A8/9) (70). The antimicrobial peptides are important in the early control and elimination *M. tuberculosis*, and together with inflammatory cytokines produced by infected macrophages can induce lung epithelial cells to produce more antimicrobial peptides.

To establish this rationale, we differentiated BMDM and infected them with *M. tuberculosis*. BMDM were also stimulated or not with IL-23 and the expression of antimicrobial peptides was determined by RT-PCR. We chose the 3, 6 and 24 hours post-infection, during which macrophages produce cytokines (as shown above): important to induce the expression of genes that encode antimicrobial peptides. We found that uninfected cells did not express the antimicrobial Lipocalin-2 whereas, infected cells, stimulated or not with IL-23, displayed increased expression of Lipocalin-2 throughout experimental infection (figure 4A). These findings were similar for LL-37/Cathelicidin where both stimulated and non-stimulated BMDM displayed increasing expression throughout infection (figure 4B).

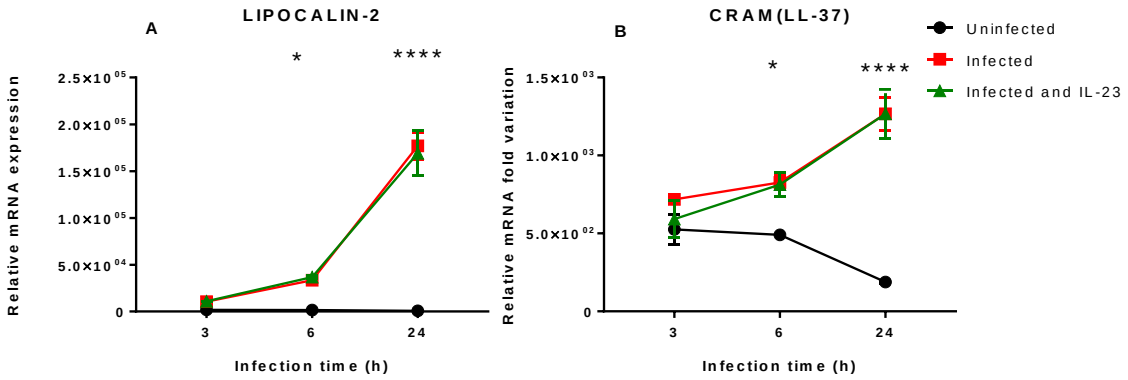

Figure 4. Innate expression of the antimicrobial peptides Lipocalin-2 and LL-37/Cathelicidin by *M. tuberculosis*-infected macrophages. Representative qPCR gene expression analysis of Lipocalin-2 and LL-37 antimicrobial peptides by *M. tuberculosis*-infected macrophages at 3h, 6h and 24h post-infection. Figure **A** showing no expression of Lipocalin-2 at 3h post-infection, but accumulation increased from 6h throughout post-infection in infected cells and cells with IL-23. While figure **B** is showing accumulation of LL-37 throughout post-infection in infected cells and infected cells with IL-23. Data representatives were analysed by 2way ANOVA with p-value, * $p = 0.0122$, **** $p< 0.0001$ for Lipocalin-2, and * $p = 0.0119$, **** $p< 0.0001$ for LL-37.

We also found that there was increased expression of the antimicrobial peptide calprotectin (S100A8) throughout infection (figure 5A and 5B). On the contrary, we found that there was a high expression of the antimicrobial peptide calprotectin (S100A9) at 3h post-infection in all cells, which increased at 6h for uninfected cells, while decreasing in infected cells stimulated with IL-23 post-infection (figure 5C and 5D).

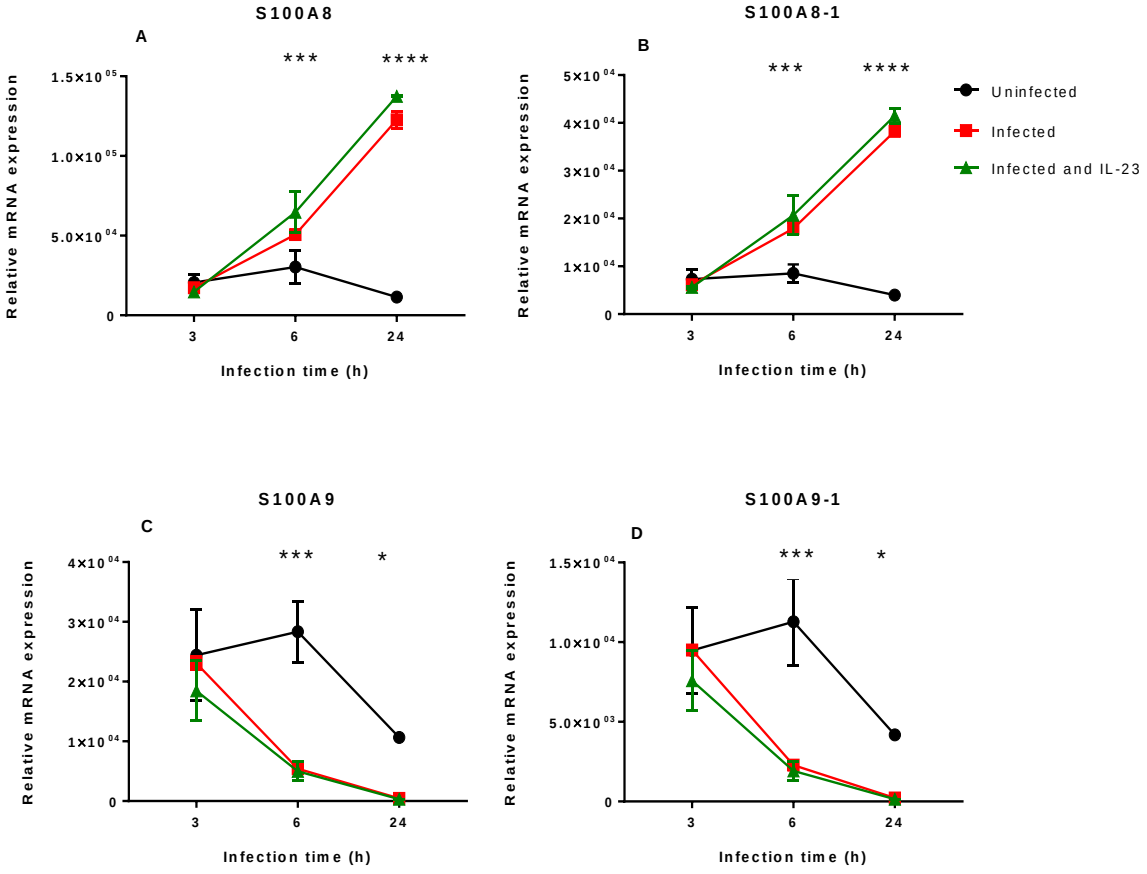

Figure 5. Innate expression of the antimicrobial peptides Calprotectins (S100A8/9) by *M. tuberculosis*-infected macrophages. Representative qPCR gene expression analysis of Calprotectins antimicrobial peptides by *M. tuberculosis*-infected macrophages at 3h, 6h and 24h post-infection. Figure **A** showing accumulation of S100A8 throughout post-infection in infected cells and cells with IL-23. While figure **B** showing decreasing accumulation of S100A9 throughout post-infection in infected cells and infected cells with IL-23. Data representatives were analysed by 2way ANOVA with p-value, *, p= 0.0002, ***, p<0.0001 for S100A8 and ***, p= 0.0003, *, p= 0.0488 for S100A9.

Taken together, these data show IL-23 does not induce the expression of antimicrobial peptides from *M. tuberculosis*-infected macrophages.

We next sought to define the role of IL-23 *in vivo*. For this, we used mice deficient in IL-23R and compared their ability to control *M. tuberculosis* with that of C57B6 mice.

Chapter II: Defining the role of IL-23 in the immune response and protection against *M. tuberculosis.*

1- Growth of *M. tuberculosis* growth in the lungs of WT and IL-23R KO mice following aerosol infection

To establish the role of IL-23 in the control of *M. tuberculosis* infection, we collected the lungs of WT and IL-23R KO mice infected through aerosol with *M. tuberculosis* H37Rv at the time points, 0, 20, 40, 80, 100 and 120 days.

At day zero (0), we performed colony forming units (CFUs) from the entire lung to confirm the dose of infection. Subsequently, CFUs were performed from lung parenchyma at day 20 to 120 pi to assess bacterial burdens. We found that, bacterial burdens increased at the same rate in both wild-type (WT) and IL-23R KO mice until day 20 pi (figure 6). Interestingly, we found that 2 out of 5 IL-23R KO mice displayed elevated bacterial burdens at day 30 post-infection, which was subsequently confirmed in the histological analysis (figure 7). From this point onwards, bacterial burdens in wild-type mice remained constant until day 120 pi. However, we found a significant increase in bacterial burdens in the lung of IL-23R KO mice at day 120 pi (figure 6). These data show that IL-23 could be playing a crucial role in the control of *M. tuberculosis* infection, since our data show that mice that are knocked for IL-23R display higher bacterial burdens during the late stages of infection. However, these findings can only be ascertained by the extent to which IL-23R KO mice affect the activation of immune cells during *M. tuberculosis* infection.

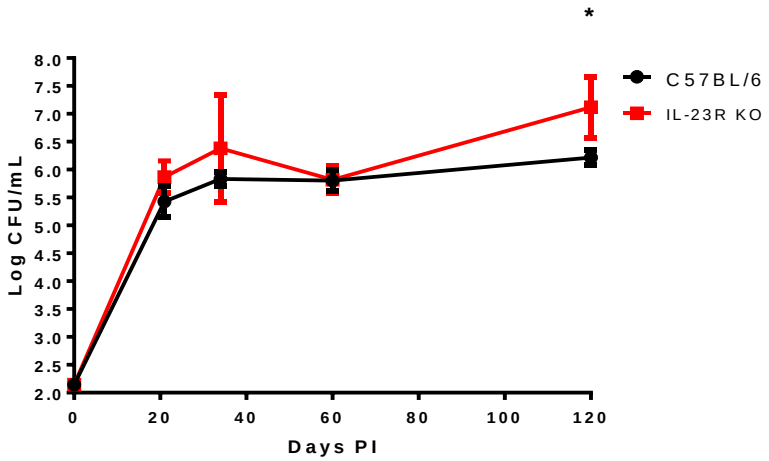

Figure 6. *M. tuberculosis* growth in the lungs of WT and IL-23R KO mice following aerosol infection. Representative growth curves of WT and IL-32R KO mice from D0 to D120 pi. The data show that WT and IL-23R KO mice had same bacterial burdens until D20, where WT could control bacterial burden until D120. However, IL-23R KO mice showed a statistically significant increase in bacterial burdens in the lung at D120 pi.

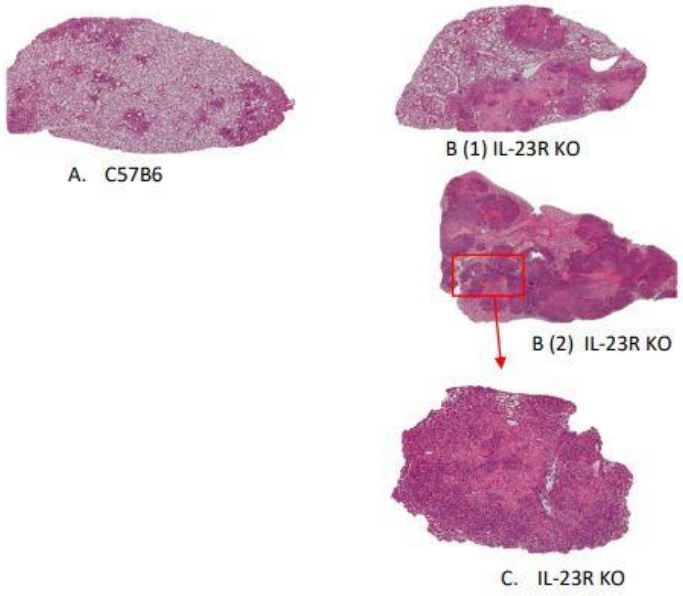

Figure 7. Histological analysis of the lung architecture of *M. tuberculosis*-infected WT and IL-23R KO mice at D30 pi. Lung parenchyma from *M. tuberculosis* infected mice were analysed by Haematoxylin and Eosin staining. **A,** showing lung architecture preserved since C57B6 mice controlled bacterial burdens, **B,** showing the lung architectures of 2 IL-23R KO mice damaged due to elevated bacterial burdens and **C,** showing a section of damaged lung parenchyma of IL-23R KO mice at D30 pi.

2- Accumulation of neutrophils in the lung of *M. tuberculosis*-infected mice

Neutrophils are important immune population that infiltrate the lung parenchyma and alveolar space during active *M. tuberculosis* infection. For this reason, we sought to determine whether neutrophils infiltration could be affected in mice deficient in IL-23R.

To assess neutrophils infiltration in IL-23R KO mice, we collected BAL fluids and lung parenchyma and performed flow cytometer analysis at days 20, 34, 60, 120.

We found that the percentages of neutrophils in BAL and lung parenchyma of both WT and IL-23R KO was relatively low at the time points analysed (figure 8A and 8B). Not surprisingly, there were relatively low numbers of neutrophils in BAL and lung parenchyma of both groups of mice at the same time points (figure 8Cand 8D). At D120 pi, we found that some IL-23R KO mice had an elevated percentage and number of neutrophils in both the BAL and lung parenchyma, possibly associated with the increased

bacterial burdens (figure 6). These data show that the absence of IL-23R does not significantly impact the accumulation of neutrophils during *M. tuberculosis* infection.

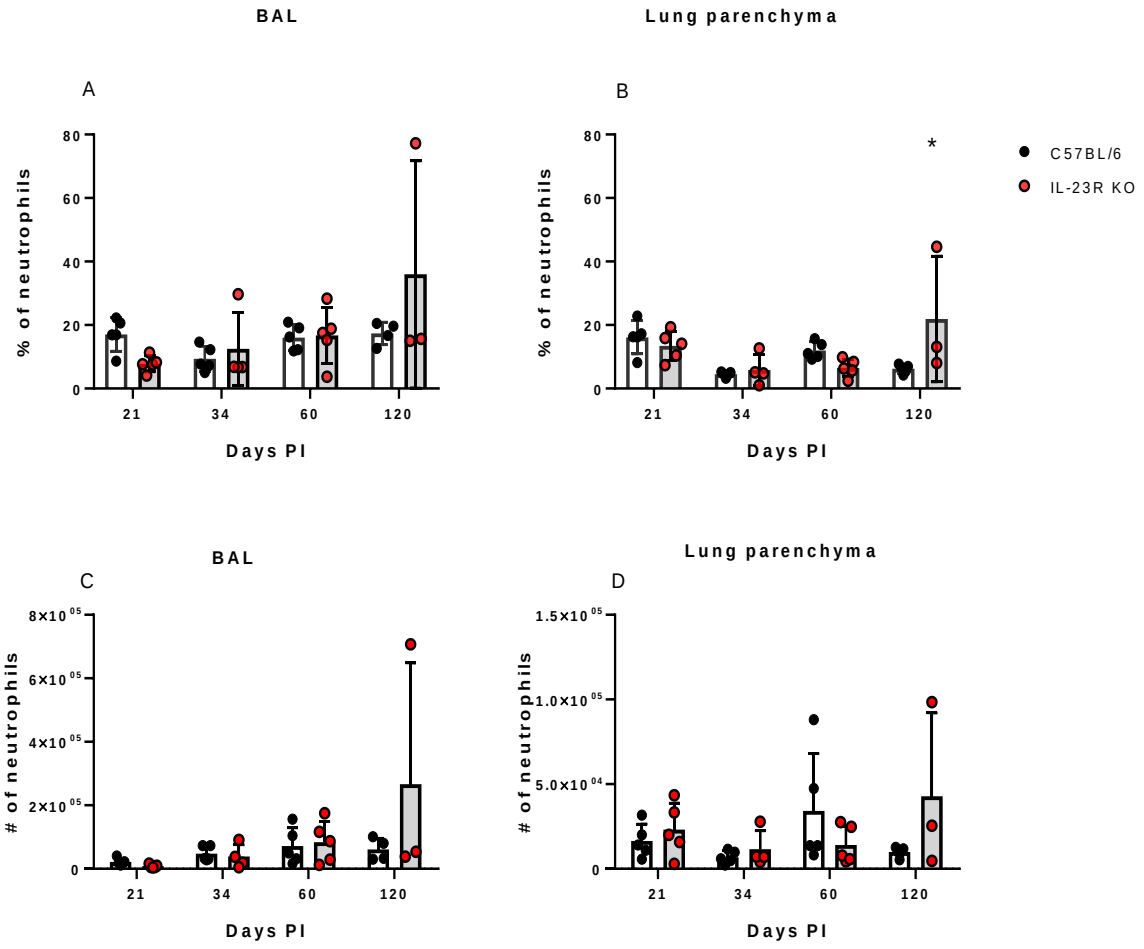

Figure 8. Accumulation of neutrophil in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for neutrophils ($CD11b^+$ Ly6G+ expression) at days 20 to 120 pi. **A**, no statistical significance of neutrophil accumulation between WT and IL-23 R KO mice, **B**, there was statistical significance at day 120 pi, IL-23 R KO showed more neutrophil expression than WT. **C** and **D**, there was no statistical significance. Data representatives were analysed by 2way ANOVA, each with five mice per group. **, $p< 0.0088$, *, $p< 0.0089$.

3.1- Accumulation of $CD4^+$ T cells in the lungs of *M. tuberculosis*-infected mice

$CD4^+$ T cells are central in the adaptive control of *M. tuberculosis* infection. Therefore, we sought to determine the kinetics of $CD4^+$ T cells in the lung parenchyma and BAL of WT and IL-23R KO mice. We collected BAL fluids and lung parenchyma from D20 corresponding to the time $CD4^+$ T cells start to infiltrate the lung, to day 120 and performed flow cytometry analysis. We found that there were similar

percentages of CD4+ T cells in BAL and lung parenchyma of both groups of mice throughout the experimental infection (figure 9A and 9B). Interestingly, higher numbers of CD4+ T cells were present in the BAL of WT mice at D34, although decreased progressively until D120 pi (figure 9C). In the lung parenchyma, we found similar numbers of CD4+ cells in both groups of mice (figure 9D). These data may point to the important role of IL-23 in the ability of CD4+ T cells to enter the BAL during the early stages of the *M. tuberculosis* infection.

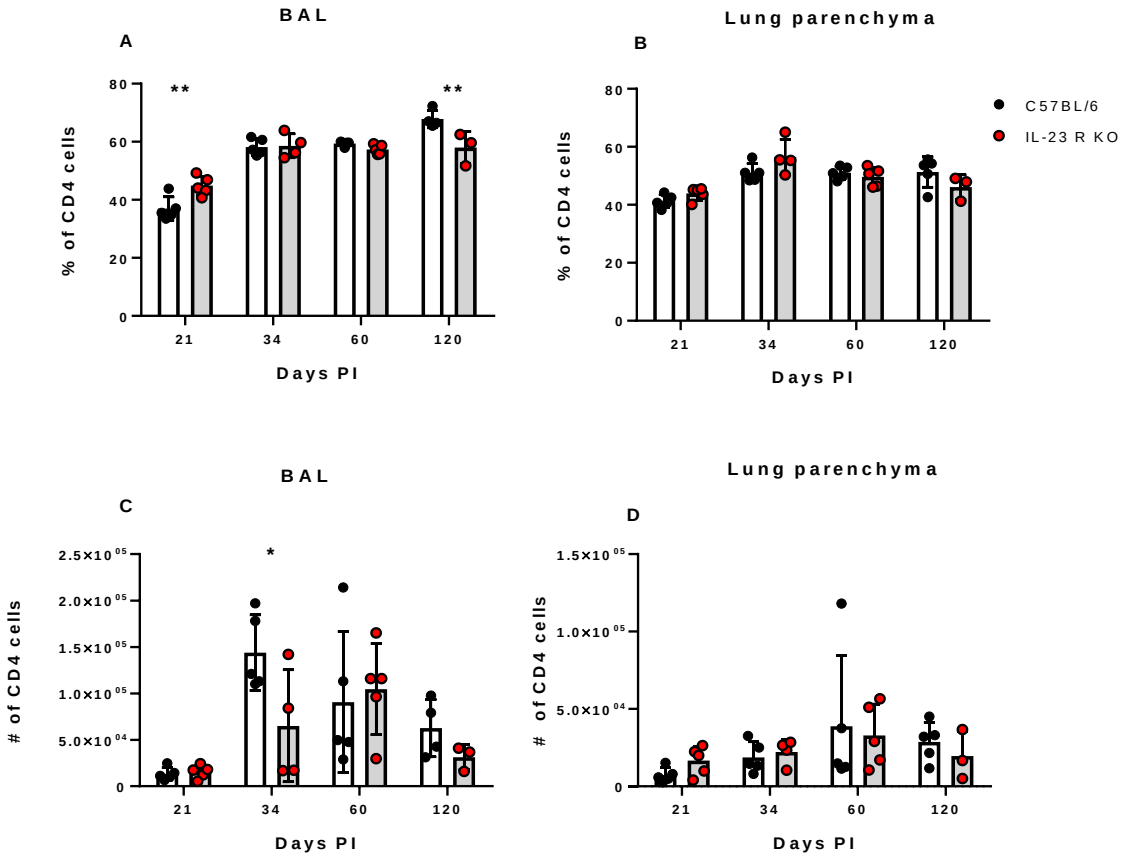

Figure 9. Accumulation of CD4+ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R OK mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD4+ T cells at days 20 to 120 pi. **A**, showing statistical significance of CD4+ T cells presence, with IL-23R KO mice showing a higher expression at days 20. Although these expressions increased progressively in both mice pi, WT showed a higher statistical significance of CD4+ T cells presence at 120 pi in BAL samples. **B,** there was no statistical significance in CD4+ T cells presence in lung samples. **C** and **D**, show no statistical significance in the numbers of CD4 T+ cells presence in BAL and lung. Data representatives were analysed by 2way ANOVA, each with five mice per group. ***, p< 0.0002; ****, p< 0.0001.

3.2- CD4+ T cells activation in the lungs of *M. tuberculosis* infected mice.

Activated CD4+ T cells during *M. tuberculosis* infection produce cytokines that activate *M. tuberculosis*-infected macrophages to kill the pathogens. Therefore, after confirming the presence of CD4+ T cells in the lung of *M. tuberculosis*-infected mice, we sought to find whether these cells were differentially activated in WT and IL-23R KO mice.

To assess this, we collected BAL fluids and lung parenchyma and analysed the expression of the activation marker CD44. As expected, we found that the percentages (figure 10A and 10B) and numbers (figure 10C and 10D) of CD4+ cells expressing this activation marker were similar in BAL and lung parenchyma of both groups of mice. Interestingly, as per the number of CD4+ T cells in the BAL described above, we also found a higher numbers of activated CD4+ T cells in the lungs of IL-23R KO mice at D34 (figure 10C). However, these data did not reach statistical significance. These data suggest that IL-23 does not impact the accumulation of activated CD4+ T cells in the lung of *M. tuberculosis*-infected mice.

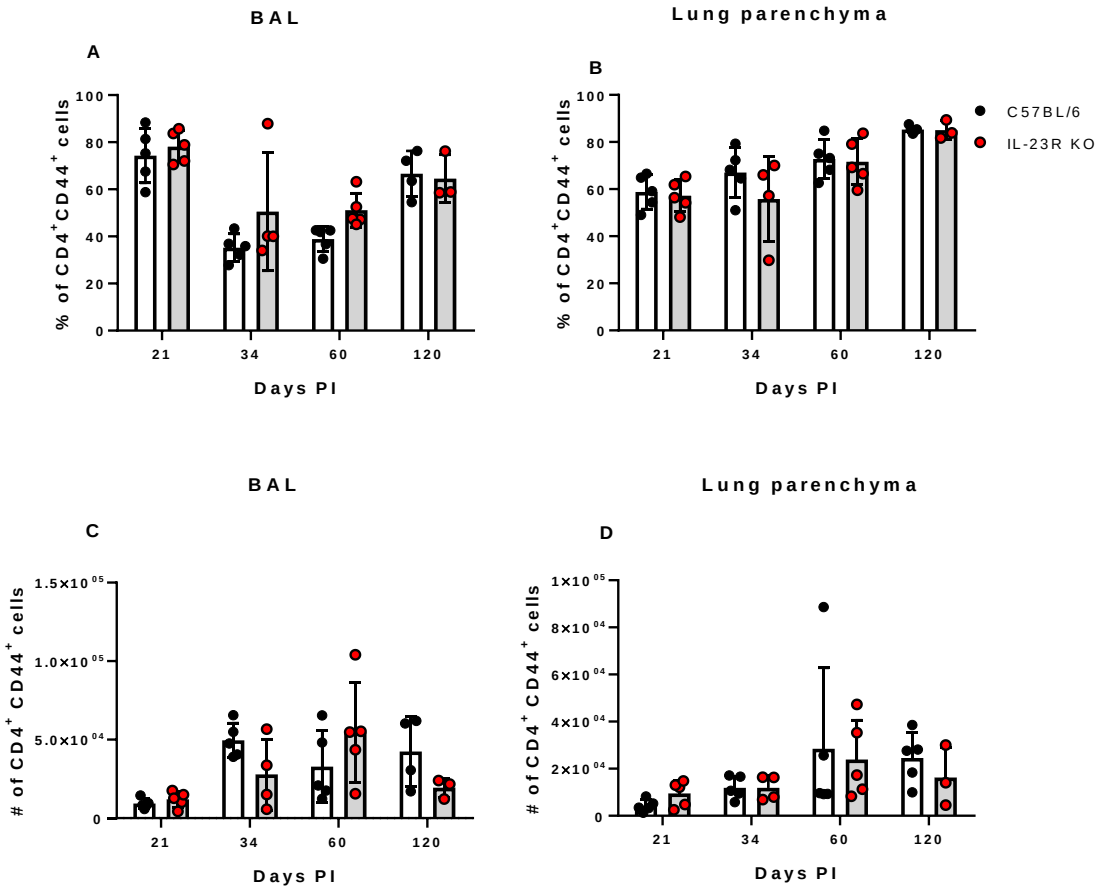

Figure 10. Activation of CD4 T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for activated CD4+ T cells at days

20 to 120 pi. **A**, showing no statistical significance of CD4+ T cells activation, **B** showing no statistical significance in CD4+ T cells activation in lung. **C,** showing no statistical of numbers of CD4+ T cells early activation and **D**, show no statistical significance. Data representatives were analysed by 2way ANOVA, each with five mice per group.

3.3- Recently activated CD4+ T cells in the lungs of *M. tuberculosis*-infected mice

With the presence of activated CD4+ cells in the lung of *M. tuberculosis*-infected mice, we sought to answer the intriguing question of whether CD4 T cells from WT and IL-23R KO mice are receiving antigen stimulation. To assess this e, we collected BAL fluids and lung parenchyma and perform flow cytometry for the early activation marker CD69. We found that there were similar percentages of CD4+ cells expressing the early activation marker in both IL-23R KO and WT mice in BAL and lung parenchyma throughout infection (figure 11A and 11B). The numbers of CD4+ cells expressing the early activation marker CD69 was similar in both groups of mice (figures 11C and 11D). We only found an increase in the percentage and number of CD4+CD69+ T cells in the BAL of IL-23R KO mice at D60, reducing significantly at D120.

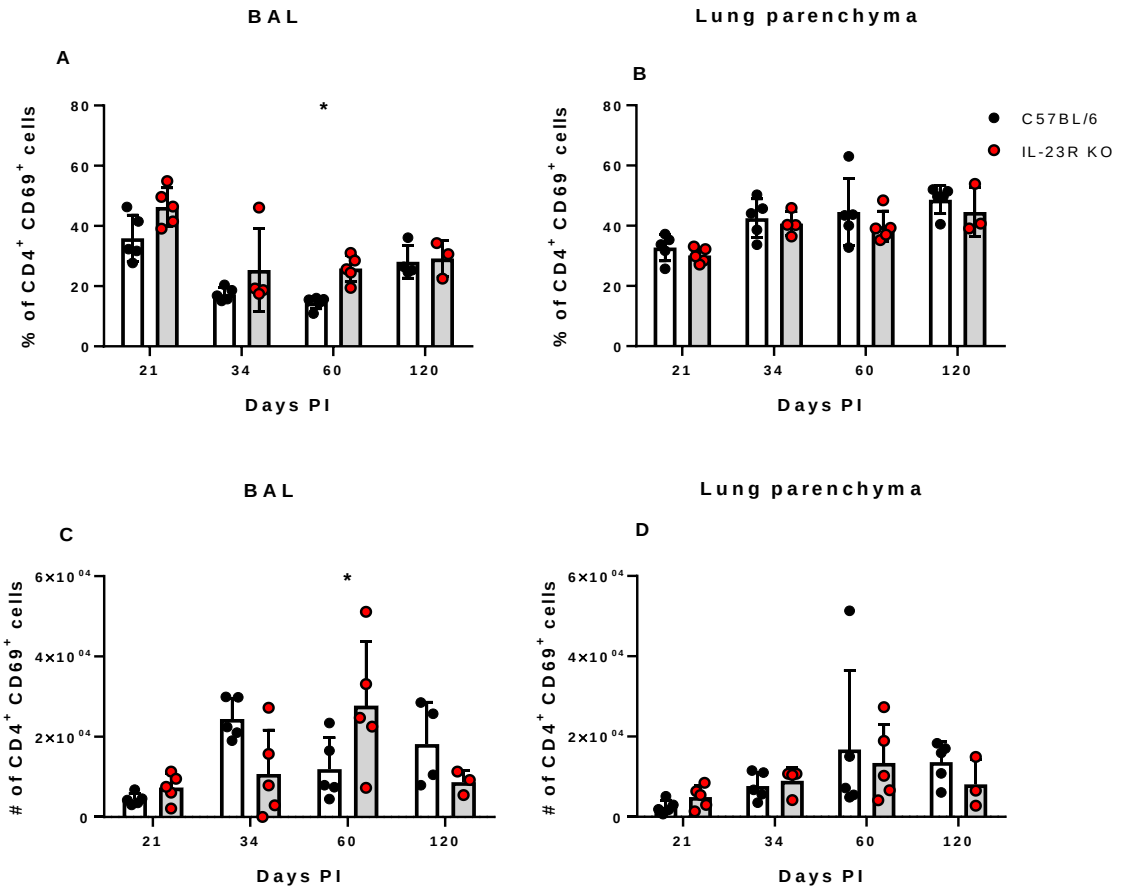

Figure 11. Recently activated of CD4+ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23RKO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD4+ T cells recently

activated at days 21 to 120 pi. **A**, showing statistical significance of $CD4^+$ T cells early activation in IL-23R KO at day 60 pi, **B** showing no statistical significance in CD4 T cells early activation in lung. **C,** showing statistical of early numbers of $CD4^+$ T cells early activation and **D**, show no statistical significance. Data representatives were analysed by 2way ANOVA, each with five mice per group. ****, $p< 0.0001$; **, $p< 0.0045$.

3.4- Regulation of $CD4^+$ T cells in the lungs of *M. tuberculosis*-infected mice

Regulatory mechanisms maintain cells in their optimal states for effector function. Therefore, we determined whether $CD4^+$ T cells expressed PD-1 differentially in the lung of *M. tuberculosis* infected WT or IL-23R KO mice. We collected BAL fluids and lung parenchyma at D20, 34, 60, 120 pi and carried out flow cytometry analysis. We found that in BAL and lung samples of both mice, the percentages of $CD4^+$ cell expressing the regulatory marker PD-1 were high at D20 pi. However, this expression was reduced by D34 to D60 and increased only IL-23R KO mice at D120 pi (figure 12A and 12B). The numbers of $CD4^+$ cells expressing PD-1 were similar in both groups of mice throughout infection (figure 12C and 12D). These data show that the absence of IL-23R activity does not influence the expression of PD-1.

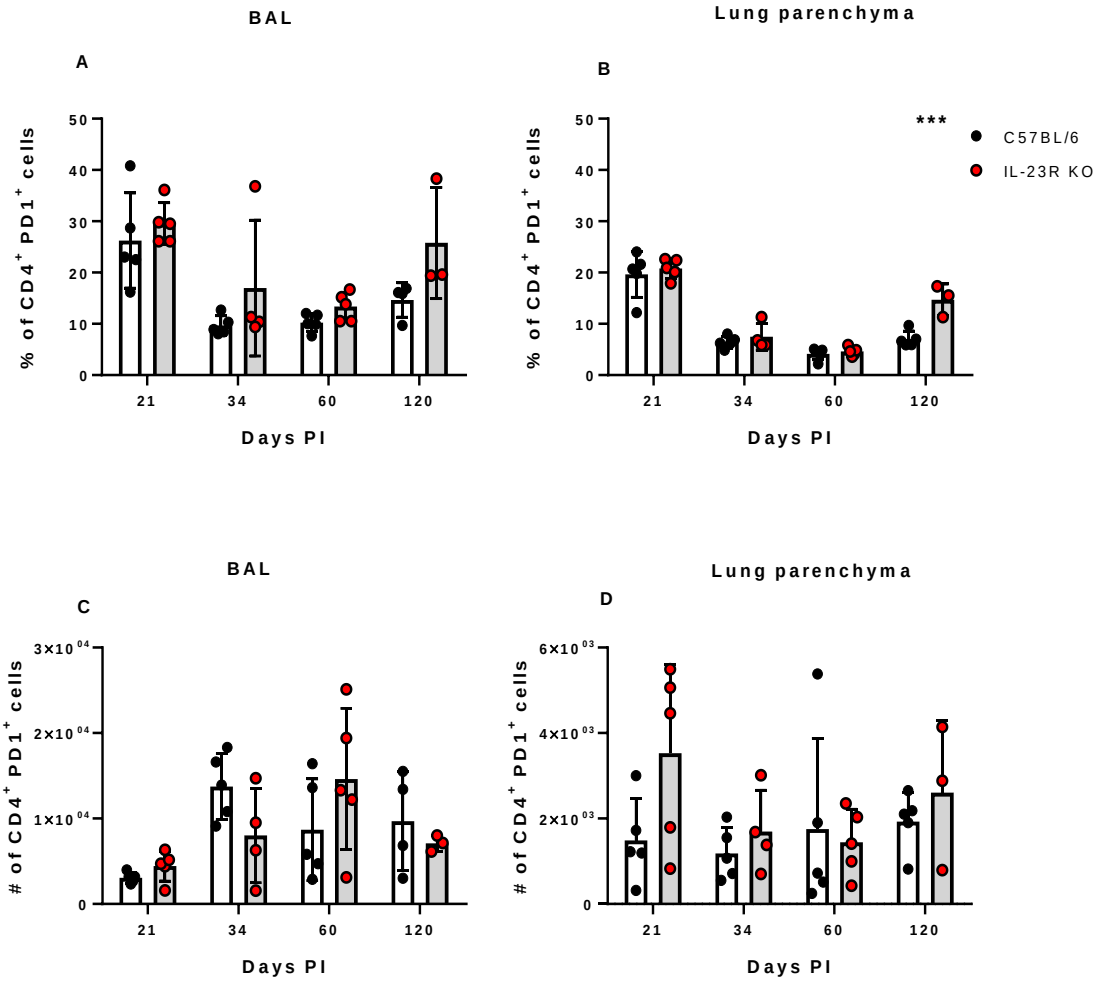

Figure 12. Expression of regulatory molecules by CD4⁺ T cells in the lungs *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD4⁺ T cells expressing PD-1 at days 20 to 120 pi. **A**, showing no statistical significance of CD4⁺ T cells regulations in BAL, **B** showing statistical significance in CD4⁺ T cells regulations at day 120 in lung of IL-23R KO mice. **C,** showing no statistical of numbers of CD4⁺ T cells early activation and **D**, show no statistical significance. Data representatives were analysed by 2way ANOVA, each with five mice per group. ***, $p < 0.0001$.

3.5- Terminal differentiation of CD4⁺ T cells in the lungs of *M. tuberculosis*-infected mice

Maturation and terminal differentiation occur CD4⁺ T cells that are chronically stimulated with antigen, impacting the effector function. Therefore, we sought to determine CD4⁺ cells undergo terminal differentiation at the same levels in WT and IL-23R KO mice. To assess this, we collected BAL fluids and lung parenchyma from D20, 34, 60, 120 pi and carried flow cytometry analysis. While we found that the percentages of CD4⁺ cells expressing the terminal differentiation marker KLRG1 were similar in BAL and

lung of WT and IL-23R KO mice (figure 13A and 13B), there were more CD4+ cells expressing KLRG1 in the BAL of the WT mice at D34 pi (figure 13C). However, these numbers reduced to normal levels at D60 pi (figure 13C and 13D).

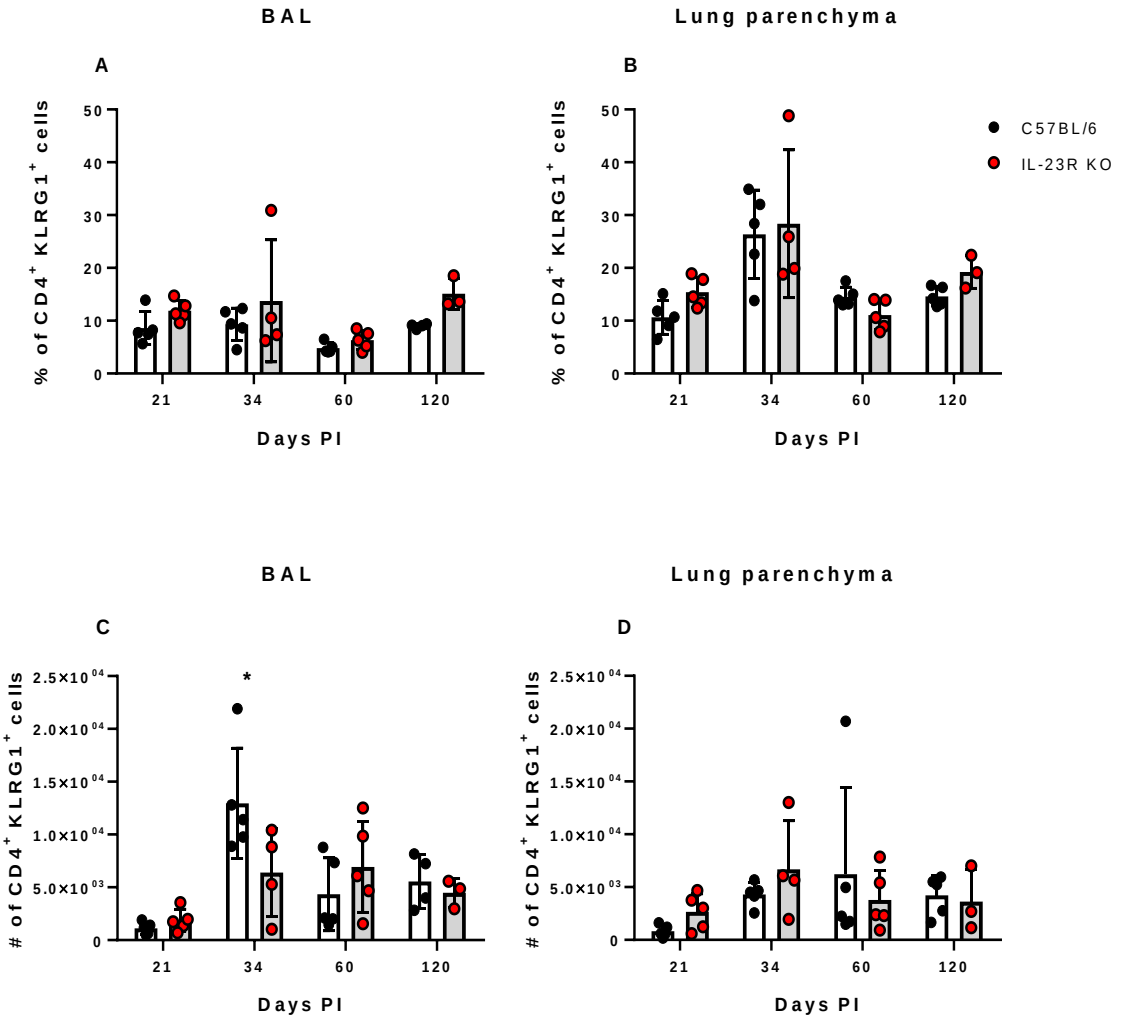

Figure 13. Terminal differentiation of CD4+ T cells in lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD4+ T cells terminal differentiation from days 20 to 120 pi. **A**, showing no statistical significance of CD4+ T cells maturation, **B** showing no statistical significance in CD4+ T cells activation in lung. **C,** showing statistical significance of numbers of CD4+ T cells maturation and **D**, show no statistical significance. Data representatives were analysed by 2way ANOVA, each with five mice per group. *, $p < 0.0365$.

From the data described above, we can conclude that the phenotypic characteristics of CD4+ T cell response is not significantly changed in the absence of IL-23R. This is an indication that IL-23R KO may

not negatively influence the effector function of $CD4^+$ T cells during *M. tuberculosis* infection. Therefore, we next investigated the $CD8^+$ T cell response in *M. tuberculosis*-infected IL-23R KO mice.

4.1- Accumulation of $CD8^+$ T cells in the lungs of *M. tuberculosis*-infected of mice

$CD8^+$ T cells are another subset of T cells that play a critical role in the control and elimination of *M. tuberculosis*, particularly during chronic stages of infection. Thus, it is imperative to investigate the role of $CD8^+$ T cells in IL-23R KO mice.

To assess the accumulation of $CD8^+$ T cells in the lung of *M. tuberculosis*-infected mice, we collected BAL fluids and lung parenchyma at D20, 34, 60, 120 pi and carried out flow cytometry analysis. We found that there were high percentages of $CD8^+$ in BAL of WT at D20 pi, which became similar in both groups of mice from D30 thereafter (figure 14A and 14B). Despite this, the numbers of $CD8^+$ cells in BAL and lung were similar in both groups of mice (figure 14C and 14D). These data show that the absence of IL-23R does not impact the accumulation of $CD8^+$ T cells.

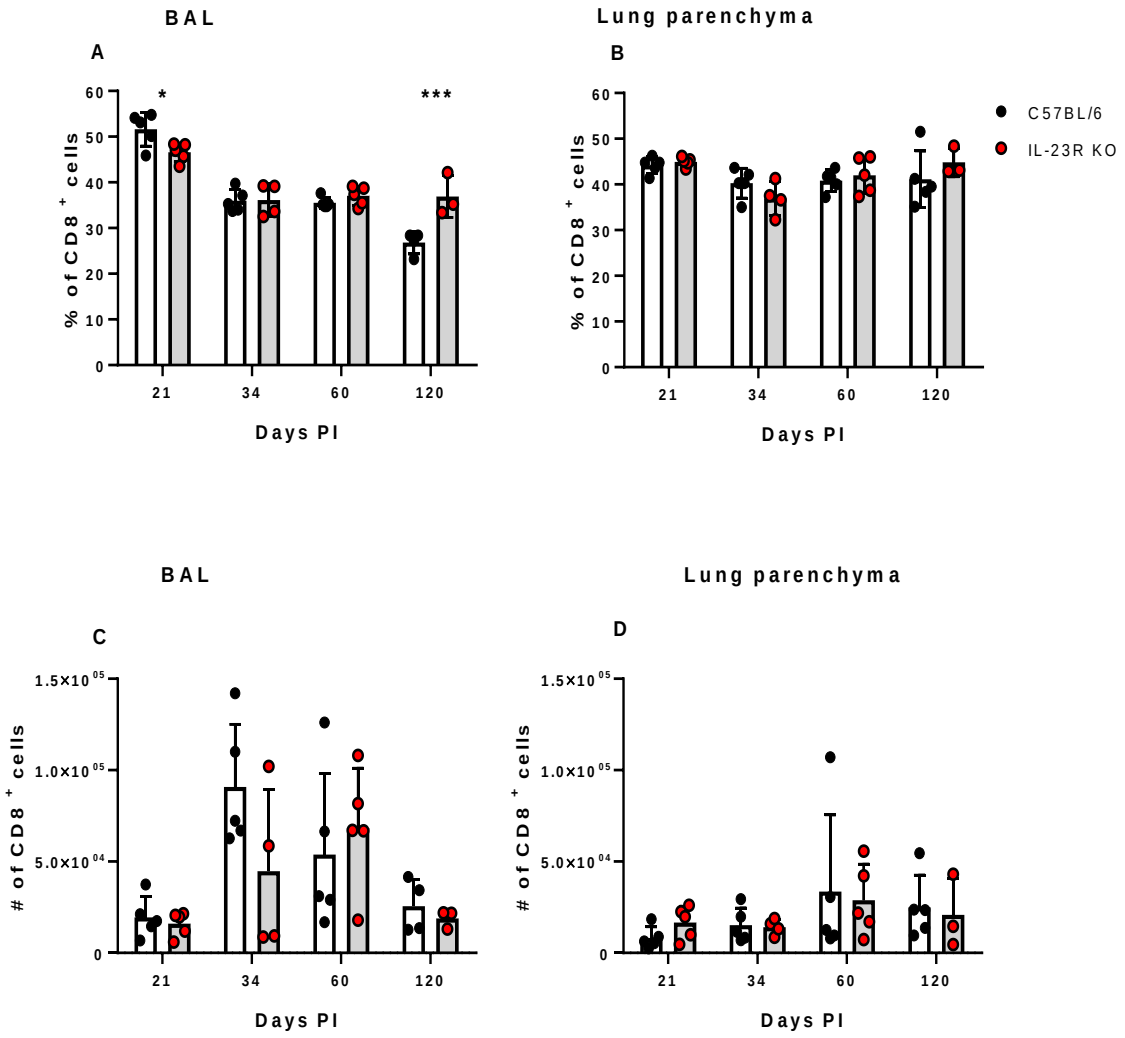

Figure 14. Accumulation of CD8⁺ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD8⁺ T cells accumulation from days 20 to 120 pi. **A**, showing statistical significance of CD8⁺ T cells expression in WT mice at day 20 pi, while at day 120 pi, there was statistical significance of CD8 T cells expression in IL-23R KO mice. **B** showing no statistical significance in CD8⁺ T cells expression in lung. **C and D** showing no statistical of numbers of CD8⁺ T cells expression. Data representatives were analysed by 2way ANOVA, each with five mice per group.

4.2- CD8⁺ T cell activation in the lungs of *M. tuberculosis*-infected mice

The data above confirm the presence of CD8 T cells in the lung of *M. tuberculosis*-infected mice. CD8⁺ T cells produce cytokines and kill *M. tuberculosis*-infected cells when stimulated or activated by *M. tuberculosis* antigens. Therefore, we sought to determine if these cells were activated in the lungs of *M. tuberculosis*-infected mice. To assess this, we collected BAL fluids and lung parenchyma at D20, 34, 60, 120 pi and performed flow cytometry analysis. We found that while the percentages of activated CD8 cells was similar in BAL of both groups of mice, there were higher percentages of activated CD8⁺ T cells

in the lung of WT mice at D34 pi (figure 15A and 15B). When we look at the number of activated CD8+ T cells, we found a reduced number of activated CD8+ T cells in the BAL of IL-23R KO mice compared to WT mice (figure 15C). The number of activated CD8+ T cells in the lung parenchyma was similar in WT and IL-23R KO mice (figure 15D).

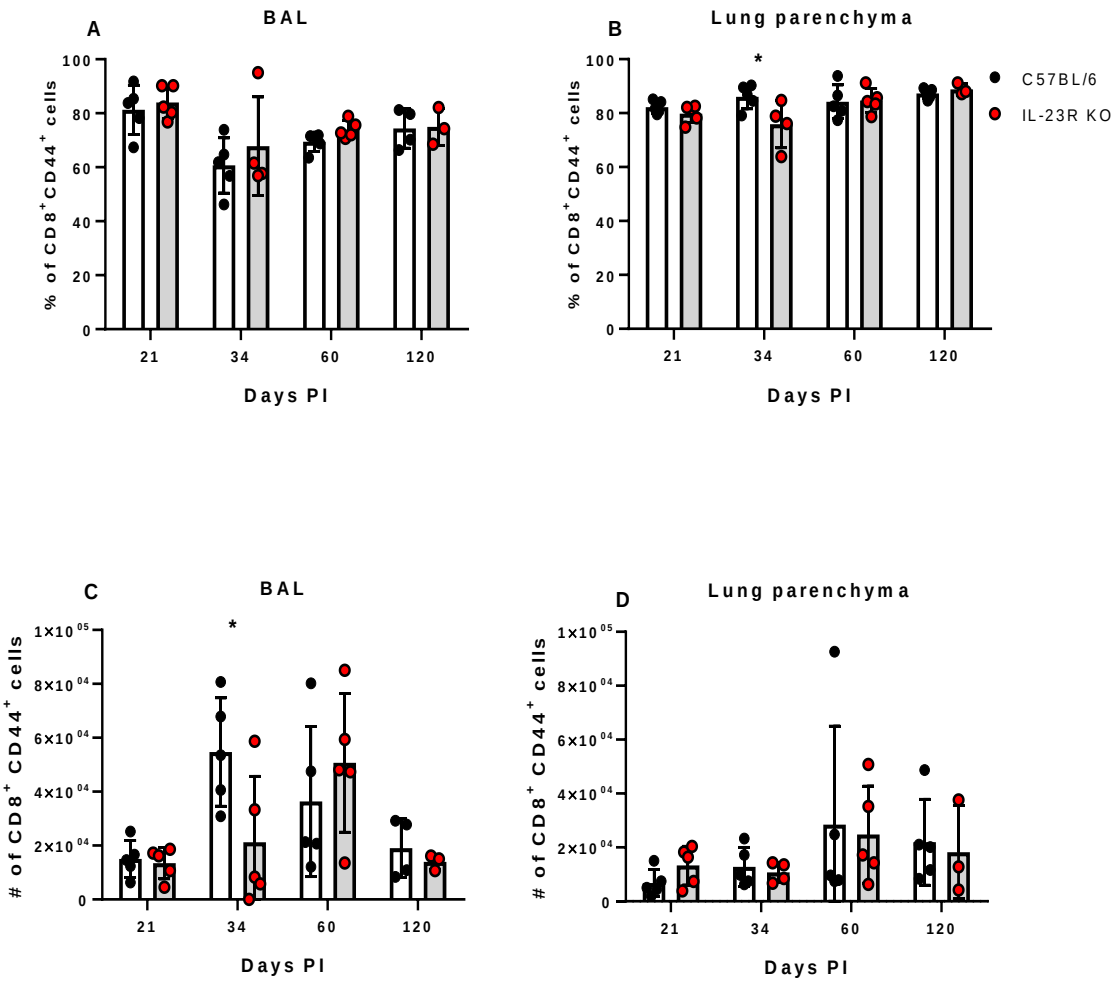

Figure 15. Activation CD8+ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD8+ T cells activation from days 20 to 120 pi. **A**, showing no statistical significance of CD8+ T cells activation, **B** showing statistical significance in CD8+ T cells activation in lung of WT mice at day 34 pi. **C,** showing statistical of numbers of CD8+ T cells activation in BAL of WT mice at day 34 pi and **D**, show no statistical significance. Data representatives were analysed by 2way ANOVA, each with five mice per group. *, p= 0.0129; *, p= 0.0035.

4.3- Recently activated CD8+ T cells in the lungs of *M. tuberculosis*-infected mice

With the presence of activated CD8+ T cells in the lung of infected mice, we sought to determine whether CD8+ T cells are receiving antigen-stimulation similarly in the lungs of WT and IL-23R KO *M tuberculosis*-infected mice. To assess this, we collected BAL and lung parenchyma at D20, 34, 60, 120 pi and carried out flow cytometry analysis. We found a higher percentage of CD8+ T cells expressing the early activation marker CD69 in BAL of IL-23R KO at D60 pi (figure 14A). At the other time points, the percentage of recently activated CD8+ T cells was similar in BAL and lung parenchyma of both groups of mice (figures 16A and 16B). In what regard the number of CD8+ cells, we found similar numbers in the BAL and parenchyma of both groups of mice (figures 16C and 16D), IL-23R KO displayed a slightly elevated number of CD69-expressing CD8+ T cells in the BAL at D60 pi (figure 16C). However, these data did not reach statistical significance.

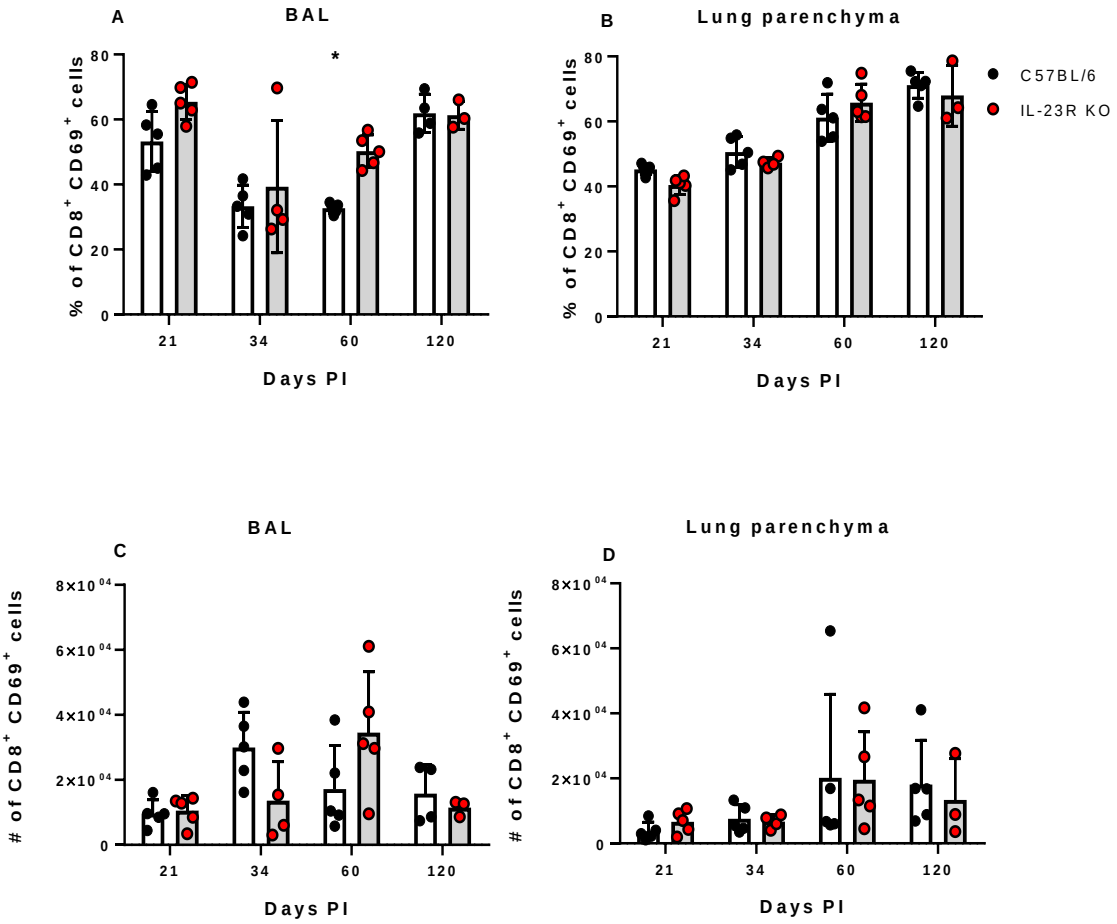

Figure 16. Recently activated CD8+ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for recently activated CD8 T cells from days 20 to 120 pi. **A**, showing statistical significance of CD8+ T cells early activation in IL-23 R KO mice

at day 34 pi, **B** showing no statistical significance in $CD8^+$ T cells early activation in lung. **C** and **D,** showing no statistical of numbers of $CD8^+$ T cells early activation. Data representatives were analysed by 2way ANOVA, each with five mice per group. *, $p < 0.0001$.

4.4- Regulation of CD8 T^+ cells in the lungs of *M. tuberculosis*-infected mice

Regulatory mechanisms play vital roles in maintaining $CD8^+$ T cells functions. Thus, we sought to determine the expression of PD-1 in $CD8^+$ T cells during *M. tuberculosis* infection. To assess this, we collected BAL fluids and lung parenchyma at D20, 34, 60, 120 pi and performed flow cytometry analysis. We found that the percentages of $CD8^+$ cells expressing the regulatory marker PD-1 was low in BAL of both groups of mice at D20 to D60 pi but increased at D120 pi (figure 17A). However, in the lung, there were relatively high percentages of $CD8^+$ cells expressing the regulatory marker PD-1 at D20 pi in both groups of mice. Although this expression was reduced at D34 pi, increased expression was observed again from D60 to D120 pi in both groups of mice (figure 17B). In line with these data, the number of $CD8^+$ cells expressing the regulatory marker PD-1 was similar in BAL and lung of both groups of mice throughout infection (figure 17C and 17D).

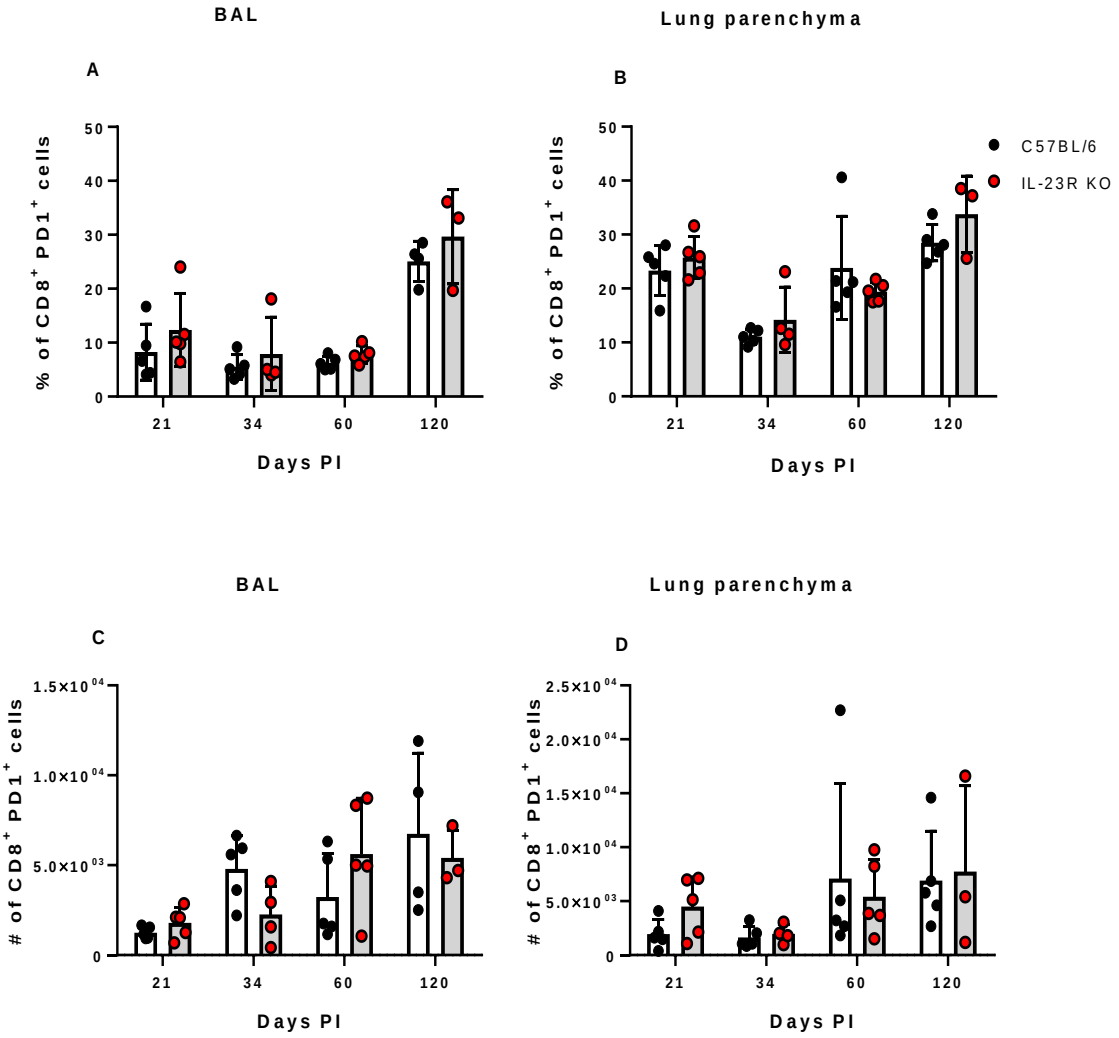

Figure 17. Regulation of CD8⁺ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry to determine the expression of PD-1 from days 20 to 120 pi. **A** and **B**, showing no statistical significance of CD8⁺ T cells regulations in BAL and lung of infected mice. **C** and **D,** showing no statistical of numbers of CD8⁺ T cells regulations in both types of mice. Data representatives were analysed by 2way ANOVA, each with five mice per group.

4.5- Terminal differentiation of CD8⁺ T cells in the lungs of *M. tuberculosis*-infected mice

Terminal differentiation can impair the protective function of CD8⁺ T cells. As such, investigating this process is important to determine the function of CD8⁺ T cells during infection. To assess this, we collected BAL fluids and lung parenchyma at D20, 34, 60, 120 pi and performed flow cytometry analysis. We found that the percentages of CD8⁺ undergoing terminal differentiation in the BAL and lung of both groups of mice was similar (figure 18A and 18B). Interestingly, the number of terminally differentiating CD8⁺ T cells

was higher at D34 in BAL of IL-23R KO mice than the WT mice; however, these data did not reach statistical significance (figure 18C and 18D).

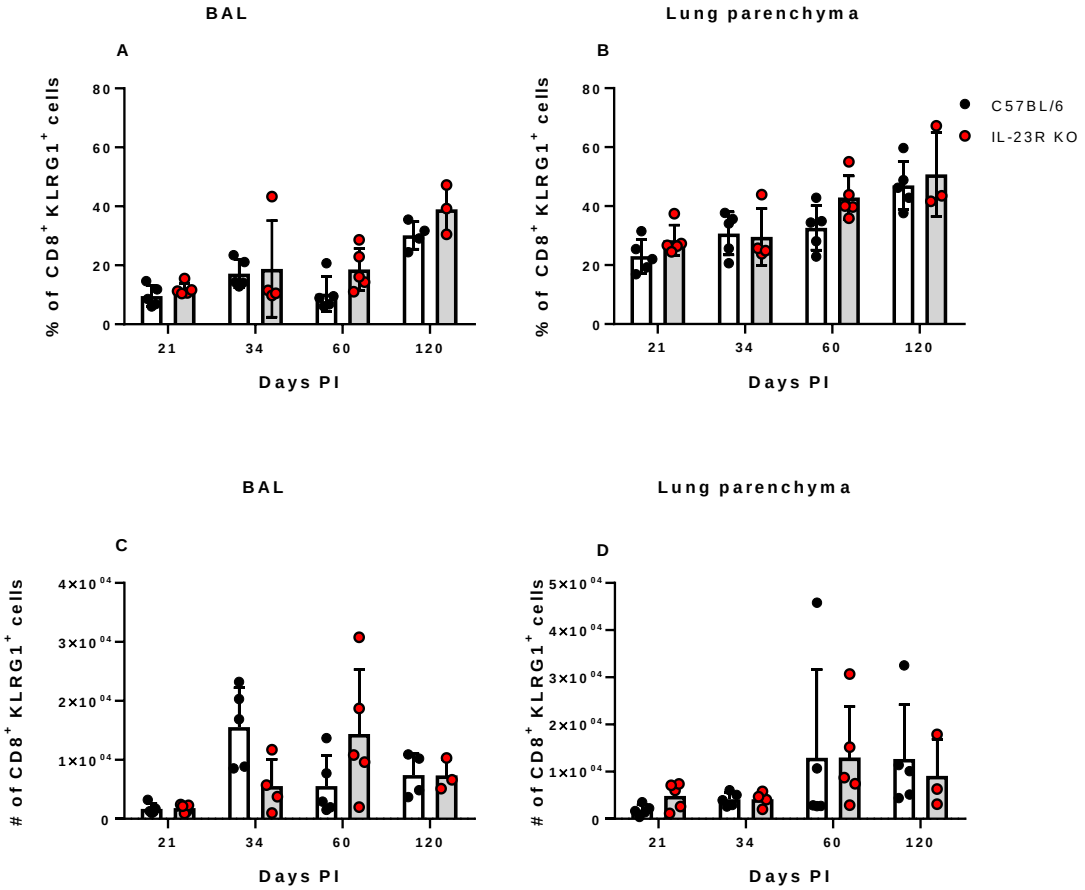

Figure 18. Terminal differentiation of CD8⁺ T cells in the lungs of *M. tuberculosis*-infected WT and IL-23R KO mice. Samples from BAL and lung parenchyma were analysed by flow cytometry for CD8⁺ T cells terminal differentiation from days 20 to 120 pi. **A** and **B** showing no statistical significance of CD8 T cells maturation and terminal differentiation in infected mice. **C** and **D**, showing no statistical significance of numbers of CD8⁺ T cells maturation. Data representatives were analysed by 2way ANOVA, each with five mice per group.

With the above data, we show the phenotypic characteristics of CD8⁺ T cells and their function in IL-23R KO mice are not significantly different from that of WT mice. As for CD4⁺ T cells, these data suggest that the absence IL-23R may not negatively influence the effector function of CD8⁺ T cells during *M. tuberculosis* infection.

Chapter III: The potential of IL-23 in enhancing protection against *M. tuberculosis* induced by mucosal vaccination

1- The role of IL-23 in protection following intranasal (i.n.) vaccination

Since our data suggest that the effective functions of T cells were not significantly affected in IL-23R KO *M. tuberculosis*-infected mice, we questioned if IL-23 could improve the efficacy of mucosal vaccination. Thus, the addition of recombinant IL-23 to BCG vaccination could improve vaccine efficacy and preserve lung tissue integrity.

To test this, we collected lung parenchyma from mice that were vaccinated through the i.n route with BCG or BCG plus recombinant IL-23 after 30 days of infection with *M. tuberculosis* and performed CFUs. Additionally, we collected the lungs of both these groups of mice and performed histology analysis. We found that mice vaccinated with BCG and mice vaccinated with BCG+IL-23 could control *M. tuberculosis* infection (figure 19A). However, when we analysed the lung tissue integrity, we found that tissue architecture of mice vaccinated intranasally with BCG+IL-23 displayed reduced areas of inflammation (figure 19B and 19C), suggesting that the activity of IL-23 during mucosal vaccination may restraint immunopathological consequences to the host.

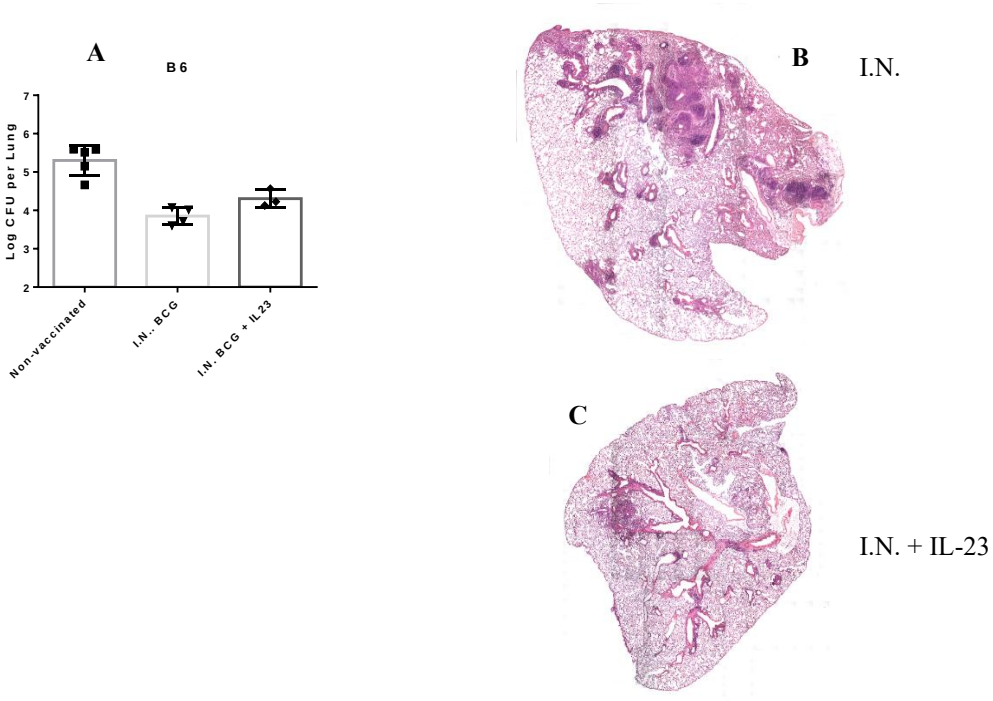

Figure 19. Potential of IL-23 in improving mucosal vaccine efficacy. Lung parenchyma were analysed for CFUs and Histology after *M. tuberculosis* infection of BCG or BCG + IL-23 vaccinated mice. **A** show that there

was no statistical significance in the control of infection between BCG and BCG with IL-23. Figures **B** and **C** showing histology of infected mice lung being preserved through vaccination.

Discussion

The importance of IL-23 in the control of *M. tuberculosis* infection is supported by the fact that mutations in the IL-12 p40 subunit and the receptor IL 12Rβ1, that is shared by IL-23 and IL-12, have previously been associated with susceptibility to mycobacteria. Importantly, a mouse model lacking the bioactive IL-12 but containing the IL-12 p40 subunit was able to mount a protective immune response against *M. tuberculosis* challenge, which suggest a role for IL-23 in protection (75). *M. tuberculosis* conserved molecular patterns with the PRR triggers the activation of diverse innate immunity mediators involved in the phagocytosis of mycobacteria and signalling pathways related to the IL-12, TNF-α, IL-6, IL-1β, IL-17, IL-23, IL-10 and TGF-β production. These cytokines have defined roles in either promoting inflammatory responses or the production of antimicrobial peptides as well as inhibiting or regulating inflammatory processes during *M. tuberculosis* infection (67).

Our data agrees with previous findings showing the early secretion of these inflammatory cytokines by *M. tuberculosis*-infected macrophages, including the proinflammatory cytokines IL-12, TNF-α, IL-6, IL-1β, IL-17 and IL-23, and anti-inflammatory cytokines IL-10 (76) and TGF-β (77). The early secretion of these cytokines is critical in the control of *M. tuberculosis* infection. TNF-α plays a major early macrophage and DCs activation, and granuloma formation (78,79,80) TNF-α also induces the production of IL-1 and IL-6, which are potent inflammatory cytokines. Indeed, while IL-6 is involved in the promotion of IFN-γ responses (76,80), IL-1β is a pyogenic cytokine with diverse inflammatory activities (82).

M. tuberculosis-infected macrophages are stimulated to secrete 12p35 and IL-23p19 that bind to IL-12p40 to form IL-12 and IL-23 respectively. The early production of IL-12p40 can drive immature dendritic cells to develop a migratory phenotype and to activate naive T cells in the draining lymph node, while IL-23p19 drive an early IL-17 response from innate cells within the lung, which has been shown to be important for control of acute bacterial infections (76,80). However, IL-10 and TGF-β play immunoregulatory and immunosuppressive functions, preventing disease exacerbation. Production of IL-10 by *M. tuberculosis*-infected macrophages may inhibit the protective immune response to pathogens by blocking the production of proinflammatory cytokines, such as TNF-α and the Th_1-polarizing cytokine IL-12, by directly acting on APCs such as macrophages and DCs (76,77,81).

The early production of IL-23 by *M. tuberculosis*-infected macrophages may induce the secretion of IL-17. The IL-23/IL-17 pathway may be playing a role in the immune response against *M. tuberculosis* infection (71).

Our hypothesis that IL-23 produced by *M. tuberculosis*-infected macrophages is critical to induce IL-17 production to stimulate the secretion of antimicrobial peptides that play vital roles in the innate control of *M. tuberculosis* infection. For example, Lipocalin-2 which prevents bacterial iron uptake by binding to bacterial siderophore (68,83), CRAMP or LL-37 that is multifunctional and immunomodulatory and increases the permeability of mycobacterial cell wall to facilitate the translocation of other antimicrobial peptides and drugs across membranes into the cytoplasm (69,84), and S100A8 and S100A9 that bind Zn^{2+} to prevent bacterial growth (70,85). However, our data show that IL-23 stimulation does not alter the antimicrobial peptide expression in *M. tuberculosis*-infected macrophages. In future experiment, we will also test the role of IL-17 in the expression of antimicrobial peptides. Additionally, we also want to determine the response of pulmonary epithelial cells to IL-23 and IL-17. Indeed, IL-17 is known to have an important role in non-hematopoietic cells in mucosal sites. As such, IL-23 and/or IL-17 sensing by epithelial cells may stimulate the expression of antimicrobial peptides by non-hematopoietic cells of the lung.

While the above *in vitro* data show that IL-23 does not induce the production of antimicrobial peptides by *M. tuberculosis*-infected macrophages, *in vivo* IL-23R KO mice show increasing bacterial burden at the late stages of the infection. It takes 20 days for sufficient antigen-specific IFN-γ-producing T cells to accumulate in the lung of a *M. tuberculosis*-infected mouse to stop bacterial growth. This is influenced by inflammatory cytokines, for example IL-12 that activates the acquired immune responses to produce IFN-γ, as well as TNF-α to control bacterial growth. However, IL-23R KO mice were also able to control bacterial growth during the early stages of infection. However, it is important to note that at D34 pi we found that some mice were unable to control *M. tuberculosis* growth and succumbed the infection. In future experiments, we will infect mice with a higher dose of *M. tuberculosis* to determine whether the ability of these mice to control bacterial burdens is maintained. If we find that IL-23R KO mice are unable to control a high dose infection, we will determine whether the impact of IL-23 is mediated through the induction of antimicrobial peptides by non-hematopoietic cells or any other unrelated mechanism.

Our bacterial burden data suggest that neither the Th_1 response nor protection is lost in the absence of IL-23, however; studies show that deficiency of IL-23R decreases IFN-γ production (87). The absence of IL-23 could be leading to ablation of the Th_{17} responses, which will result in a substantial loss of IL-17 response in the lung following mycobacterial infection. It is possible that TGF-β produced by *M. tuberculosis*-infected DCs could be inducing the differentiation of Th_{17} from naïve $CD4^+$ T cells (86), although these Th_{17} cells could not be stimulated to produce IL-17 for the control of bacterial growth due

to the absence of IL-23 (41). One possible effect of this reduced Th_{17} response is a reduced neutrophil accumulation. However, our data revealed there are low numbers of neutrophils in the lung of *M. tuberculosis*-infected WT and IL-23R KO mice. As discussed above, a higher dose infection may also highlight the impact of IL-23 in the neutrophil population in the lungs of *M. tuberculosis* infected mice. Neutrophils are a major cell type that are recruited in the lung during active *M. tuberculosis* infection and greatly contribute to control of *M. tuberculosis* infection due to their ability to produce antimicrobial peptides (11,14), although intense neutrophilia is an important factor contributing to inflammatory immunopathology (11,14,65). There is evidence of a direct relationship of Th_{17} and neutrophil recruitment in *M. tuberculosis* infection, and this showed that both IL-17 production and neutrophil recruitment have been found in *M. tuberculosis* infection. IL-17-mediates recruitment of neutrophils through induction of chemokine and cytokines secretion by epithelial and endothelial cells (65). Our data showing very low neutrophil accumulation from days 21 to 60 could be due to absence of IL-23 in *M. tuberculosis*-infected mice IL-23R OK. The absence of IL-23 could be resulting in low production of IL-17 to induce neutrophil recruitment. However, the rise of neutrophils at day 120 could be due to a second wave of neutrophil recruitment, the adaptive wave that reached the lung together after IFN-γ and IL-17-producing T cells (65). The low neutrophil accumulation in the WT-infected mice could be probably due to the fact the bacterial burden is contained by T cells.

T-cell responses are central for the protection against *M. tuberculosis* infection, primarily because this pathogen lives intracellularly in cells (88). A mouse model for *M. tuberculosis* has shown that IFN-γ produced by both $CD4^+$ Th_1 cells and $CD8^+$ T cells is key for the protection against *M. tuberculosis* infection and for human immune control of *M. tuberculosis* infection (88). Distinct populations of T helper cells differ by the expressed cytokines and transcription factors and by their response to the pathogen. These are induced upon infection with this intracellular pathogen and mediate protection mainly by activating macrophages to kill *M. tuberculosis* (45). Our data show the activation of $CD4^+$ T and $CD8^+$ T cells in both the WT and IL-23R KO *M. tuberculosis*-infected mice. The induction of Th_1 responses could be through the cytokine IL-12 produced by antigen-presenting cells, which Interacts with the IL-12 receptor expressed on the surface of T cells (45,89) and recognition of peptides through the cytosol (88). Although we found activated T cell responses following *M. tuberculosis* infection, due to the expression of CD44 by T cells (90,91,92), we could confirm that these cells were being stimulated in the lung by *M. tuberculosis* antigens as they express the early activation marker CD69 (93,94). Our findings are also in agreement with recent studies showing the effector functions of T cells are maintained during *M. tuberculosis* infection through prevention of T cells exhaustion. T cells exhaustion is prevented by the expression of

the surface marker PD-1 for the optimal production of IFN-γ. Blockade of PD-1 receptor could be one of the strategies to revert T-cells from exhaustion during *M. tuberculosis* infection (95,96). Furthermore, T cells could be undergoing constant differentiation, enabling them to become more specialized in their effector functions against *M. tuberculosis* infection, through the expression of KLRG1 (97,98).

IL-23 could be used to improve vaccine efficacy through the IL-23/IL-17 pathway. IL-23 release by antigen-presenting cells triggers rapid IL-17 responses from tissue-resident T cells and drives the development of IL-17-producing T cells, the Th_{17} cells. IL-17 impacts inflammatory responses to *M. tuberculosis* in the lung, suggesting a protective role of these cytokines. Therefore, IL-23 drives early immune responses to *M. tuberculosis* through IL-17 induction (40,41,99). Studies report on mouse models show that Local delivery of IL-23 gene stimulates expression of both IFN-γ and IL-17 in lung tissues. Thus, when administered prior to infection with *M. tuberculosis*, IL-23 gene reduced bacterial burden because of elevated numbers of activated T cells in lungs and draining lymph nodes (99). Our data agree with these findings that prior intranasal vaccination of mice with BCG and BCG with IL-23 could be stimulating the expression of IFN-γ and IL-17 in the lung, which controlled *M. tuberculosis* aerosol infection. Indeed, while we found similar bacterial burdens in BCG or BCG+IL-23 vaccinated mice, the later displayed reduced inflammatory lesions of the lung. Future research will be important to define the mechanisms whereby IL-23 is promoting a less pathological response, without impacting bacterial burdens.

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