Understanding the modulation of myeloid cell responses by clinical isolates of *Mycobacterium tuberculosis* associated with mild or severe tuberculosis

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Dissertação realizada no âmbito do Mestrado em Bioengenharia

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Agosto, 2022

Resumo

A tuberculose (TB) é uma doença causada pelo agente patogénico *Mycobacterium tuberculosis* (*Mtb*). Altamente disseminada no Sudeste Asiático e África Subsariana, a TB tem sido alvo de negligência nos dias de hoje, não só devido ao abrupto surgimento da pandemia Covid-19, mas também à diminuição dos recursos alocados à sua investigação científica. Um "gigante adormecido", latente em aproximadamente ¼ da população mundial, *Mtb* evoluiu ao longo do tempo modulando de forma diferenciada as interações estabelecidas com o hospedeiro. Na verdade, a severidade da TB depende de vários fatores, nomeadamente de características intrínsecas à própria bactéria. Dois isolados clínicos de *Mtb* estudados no nosso grupo, 4I2 e 6C4, apresentam no modelo de ratinho diferenças em termos de crescimento pulmonar, modulação de interleucina (IL)-1β e recrutamento de neutrófilos. O isolado 4I2 promove uma alta indução de IL-1β, enquanto infeções pelo isolado 6C4 se traduzem em doença mais severa, maior taxa de replicação pulmonar, baixa indução de IL-1β e recrutamento persistente de neutrófilos com resposta exacerbada de IL-17.

Com o intuito de explicar os mecanismos subjacentes a estas diferenças entre os isolados 4I2 e 6C4, debruçámo-nos sobre o papel desempenhado pela sinalização do recetor de IL-1 (IL-1R) no compartimento mielóide e na influência de IL-23 na geração de respostas exacerbadas de IL-17, usando modelos *in vitro* de macrófagos alveolares, monócitos e células dendríticas e modelos *in vivo* de ratinhos deficientes para o recetor de IL-23 (IL-23R$^{-/-}$). Macrófagos alveolares e monócitos revelaram-se permissivos à replicação dos isolados 4I2 e 6C4, dependendo da sinalização de IL-1R para regular a expressão genética e secreção proteica da sua rede inflamatória. Células dendríticas afiguraram-se como fortes produtoras de mediadores inflamatórios quer perante *Mtb* 4I2, quer perante *Mtb* 6C4, dependendo da sinalização de IL-1R para conter o crescimento bacteriano e regular a expressão genética da sua rede inflamatória. Por sua vez, a sinalização de IL-23R não parece explicar a contínua infiltração pulmonar de neutrófilos em infeções por *Mtb* 6C4, ainda que a sua ausência leve ao estabelecimento de um microambiente inflamatório aparentemente mais benéfico para o hospedeiro.

Palavras-chave: tuberculose, *Mycobacterium tuberculosis*, macrófagos alveolares, monócitos, células dendríticas, neutrófilos, IL-1, IL-23

Abstract

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (*Mtb*). Highly disseminated in Southeast Asia and Sub-Saharan Africa, TB is largely neglected in our society, not only due to the rapid emergence of the Covid-19 pandemic, but also because of a lower allocation of resources to its research. A "sleeping giant", latent in about ¼ of the entire global population, *Mtb* evolved throughout time differentially modulating host-pathogen interactions. In fact, the severity of TB is dependent on several factors, namely bacterial intrinsic characteristics. *Mtb* 4I2 and 6C4, two clinical isolates studied within our group, display differential bacterial replication rates, interleukin (IL)-1β modulation and neutrophil recruitment in the mouse model of *Mtb* infection. *Mtb* 4I2 leads to high IL-1β induction, whereas *Mtb* 6C4 infections yield low IL-1β induction, more severe disease, higher bacterial burdens and increased lung neutrophil influx with exacerbated IL-17 responses.

We attempted to unravel the mechanisms behind these differences by looking at the role of IL-1 within the myeloid compartment and the influence of IL-23 in the establishment of IL-17-driven responses, using *in vitro* models of alveolar macrophages, monocytes and dendritic cells and *in vivo* ones of mice with deficiencies in the IL-23 receptor (IL-23R$^{-/-}$). Alveolar macrophages and monocytes were permissive to *Mtb* 4I2 and 6C4, relying on IL-1 receptor (IL-1R) signalling to regulate gene expression and cytokine production. Dendritic cells were shown to be higher producers of inflammatory mediators in response to both isolates 4I2 and 6C4 and rely on IL-1R signalling to contain bacterial replication and establish a balanced inflammatory gene network. Furthermore, we found that the IL-23R signalling does not appear to explain the observed lung neutrophil accumulation within *Mtb* 6C4 infections, although its absence leads to an apparently more favourable host inflammatory environment.

Keywords: tuberculosis, *Mycobacterium tuberculosis*, alveolar macrophages, monocytes, dendritic cells, neutrophils, IL-1, IL-23

Agradecimentos

Queria prestar o meu profundo reconhecimento e gratidão às seguintes pessoas:

Primeiramente, à minha orientadora Margarida Saraiva, pela confiança que me foi atribuída e por me ter facultado todas as ferramentas necessárias à elaboração desta dissertação.

Seguidamente, ao grande Immune Regulation, pela calorosa receção e estabelecimento de um grupo tão unido. À Rute Gonçalves, Joana Couto, Joana Coelho, Ana Fernandes, Marta Silva, Rita Santos, Tânia Lima e Catarina Martins, mas muito particularmente ao meu orientador oficioso, Diogo Silvério, pelo acompanhamento constante e transmissão de conhecimentos em âmbito P3. Agradeço também ao ICVS na pessoa do Egídio Torrado e da Consuelo Micheli.

Agradeço igualmente aos meus amigos, quer beirões quer do município mais setentrional do nosso país.

Finalmente, à minha família. Ao meu irmão, meu eterno mentor, bem como aos meus pais, pelo inestimável apoio, sacrifícios constantes e serem sem sombra de dúvida a base mais importante da minha vida.

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AM	Alveolar macrophage
AMP	Antimicrobial peptide
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette Guérin
BMDM	Bone marrow-derived macrophages
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming-unit
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESAT	Early secretory antigen target 6
ESX	ESAT-6 secretion system 1
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony stimulating factor
HAX-1	HS-1-associated protein X-1
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IGRA	Interferon-gamma release assay
IL	Interleukin
iNOS	inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
JAK	Janus kinase

LN	Lymph node
LPS	Lipopolysaccharide
LTBI	Latent tuberculosis infection
Lyz	Lysozyme
MHC	Major histocompatibility complex
mL	Millilitre
MOI	Multiplicity of infection
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
Myd	Myeloid differentiation primary response
NET	Neutrophil extracellular trap
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor erythroid 2-related factor 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
qPCR	Quantitative polymerase chain reaction
Siglec	Sialic acid-binding immunoglobulin-like lectin
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
T-bet	T-box expressed in T cells
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
TRAF	Tumor necrosis factor receptor associated factor
TST	Tuberculin skin test
WT	Wild type

Chapter 1 – Introduction

Epidemiology

Tuberculosis (TB) is the 2nd leading infectious and killing disease, only surpassed by Covid-19[1,2]. In 2020, approximately 10 million new active TB cases emerged and 1.5 million people succumbed to the disease[3,4]. One quarter of the global population is estimated to have been exposed to the pathogen, therefore being considered latently infected[5]. The incidence and prevalence of TB are mainly reported in developing regions, with Southeast Asia (43%), Africa (25%) and Western Pacific (18%) comprising the majority of cases[6] (**Fig. 1**).

The currently employed Bacillus Calmette-Guérin (BCG) vaccine, despite being effective in the protection from disseminated disease, does not prevent infected adults from developing pulmonary TB[7]. The evolution of multi-drug resistant bacteria[8,9,10], the exacerbated impact that HIV co-infections disproportionately have in disadvantaged populations[11] and the recent reduction in annual global investments allocated for TB research[6] make this disease a growing matter of concern. Therefore, awareness needs to be raised about the need of improving TB prevention, diagnosis, control and treatment.

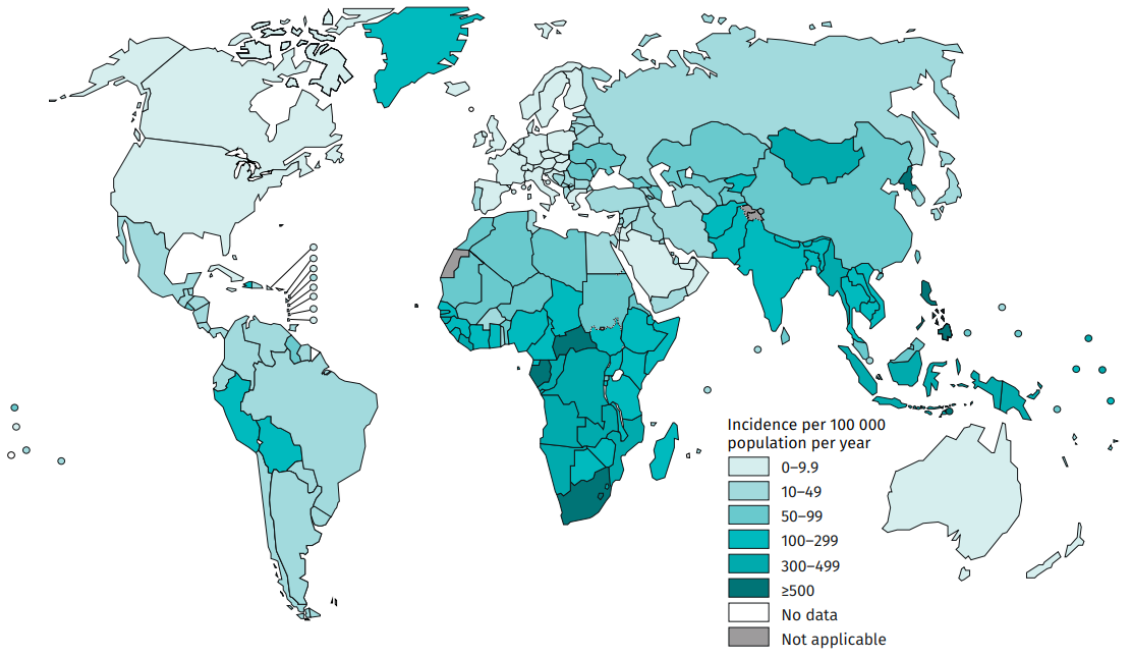

Fig. 1 TB incidence in 2020. From "Global tuberculosis report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO."

Taxonomy, disease and transmissibility

TB is caused by infection with the bacillus *Mycobacterium tuberculosis* (*Mtb*), an airborne bacteria belonging to the actinomycetales order and mycobacteriaceae family[12,13]. *Mtb* is a slow-growing, gram-positive, acid-fast bacilli[14] that transmits through inhalation of droplets containing bacilli expelled from a TB patient[15,16]. Upon reaching the respiratory tract, there are three major possible outcomes for the encounter between the host organism and the bacteria[17,18,19]: i) pathogen elimination through mechanisms likely involving innate immunity (**Fig. 2a**); ii) bacterial containment with persistent immune response – in which *Mtb* infection is latent (LTBI) and the individual has no clinical manifestations neither ability to spread the pathogen (**Fig. 2b**); iii) development of active disease – in which the individual is symptomatic and may transmit disease (**Fig. 2c**). The ability of the pathogen to transit between latency and activation demonstrates how dynamic the spectrum of TB states really is[20]. In fact, an estimated 10% of latent cases eventually progress to active TB, a risk which is seriously aggravated upon aging[21] and the presence of co-morbidities[22]. Since in this thesis we focus on the development of disease, the following sections will only cover active TB disease.

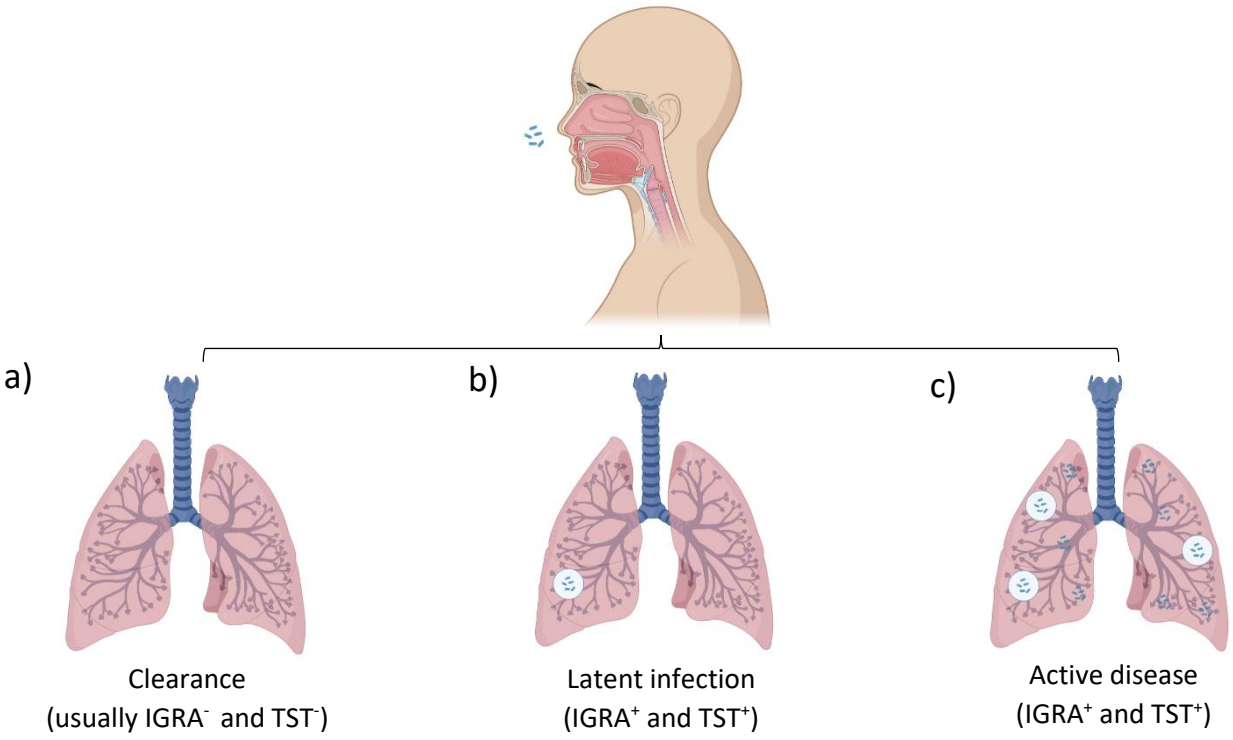

Fig. 2 TB disease states. *Mtb* can be immediately cleared following infection (a), contained within granulomas and persist in a latent state (b) or progress to active disease while disseminating throughout the lungs (c). A latent infection can be diagnosed recurring to IFN-γ release assays (IGRA) and tuberculin skin tests (TST). The former measures the titres of IFN-γ released from T cells following stimulation with *Mtb*-specific antigens, while the latter assays the extent of a dealyed-type hypersensitivity response upon injection with tuberculin purified protein derivative. Individuals who overcome infection likely without the intervention of T cell responses remain IGRA$^-$ and TST$^-$, while those with active disease are IGRA$^+$ and TST$^+$. Nonetheless, if a strong memory T-cell response is kept after pathogen clearance, an individual may still be IGRA$^+$ and TST$^+$.

Granuloma – a dynamic spectrum

The clinical hallmark of TB disease is the granuloma – an immune cell aggregate composed of a macrophage centre surrounded by a peripheral cuff comprised of fibroblasts, neutrophils, dendritic cells (DCs), T cells and B cells[23,24]. These structures are initiated when alveolar macrophages (AMs) engulf *Mtb* bacilli and migrate into deeper sites in the lung, arresting bacterial growth with microbicidal mechanisms such as TNF-α production[25]. The central core of the granuloma is formed by these infected macrophages and other macrophages which differentiate into multinucleated giant, epitheloid and foamy cells[25]. The recruitment of additional neutrophils, IFN-γ-producing T cells and fibroblasts further contributes to the expansion of the granuloma and enhances its microbicidal capacity[26].

Structurally, granulomas are heterogeneous and can be divided in three main different subsets, which are differentially associated with disease severity[27,28]: i) caseous, with a central hypoxic and necrotic area composed of dead macrophages, in which *Mtb* is able to reside in a latent way with altered metabolic states (**Fig. 3a**); ii) non-necrotizing, usually found in active disease, are essentially composed by macrophages and very few lymphocytes (**Fig. 3b**); iii) restrictive, mainly constituted by fibroblasts, are associated with disease clearance (**Fig. 3c**).

The established host-pathogen interactions and immune system waning influence the balance between pro and anti-inflammatory mediators, which alters the granuloma trajectory and further progress of LTBI to active TB[29]. For instance, excessive TNF-α and IFN-γ signalling incur in macrophage necrosis and *Mtb* extracellular release[26]. Once the pathogen breaks out of the granuloma and erodes vascular barriers, leading to cavitation, bacilli spillage and bacteria dissemination throughout the body, *Mtb* transmission cycle is reinitiated[30].

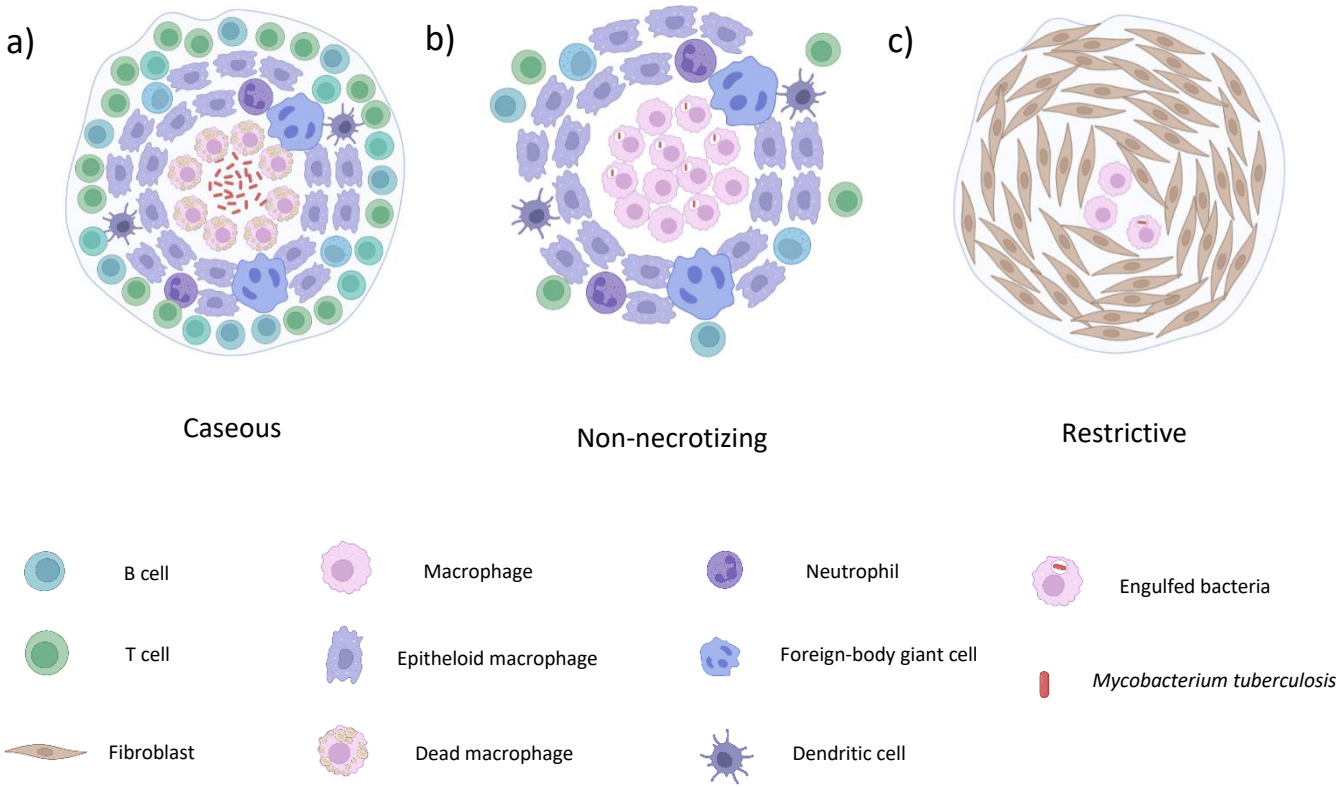

Fig. 3 Structure and composition of different types of granuloma in infections with *Mtb*. Adapted from: Barry, C., Boshoff, H., Dartois, V. et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol* 7, 845–855 (2009). https://doi.org/10.1038/nrmicro2236

Immune response to *Mtb* – a brief overview

Infection with *Mtb* begins once AMs, the first line of defence against TB, encounter and engulf bacteria[19,31] (**Fig. 4**). The recognition of *Mtb* is mediated by a wide array of receptors, including scavenger, complement and mannose receptors[25]. Beyond the recruitment of macrophages, neutrophils, monocytes and DCs, AMs also participate in *Mtb* elimination through upregulation of several microbicidal mechanisms: i) autophagy; ii) phagosome acidification; iii) production of oxygen components, antimicrobial peptides and the inflammatory cytokines TNF-α, IL-12, IFN-γ and IL-1β[32].

Out of the initially recruited cells, DCs establish the link between innate and adaptive immunity, as they are able to phagocyte bacteria and travel to the lymph node (LN), priming $CD4^+$ naïve T cells to differentiate either into a T helper (Th)1 or Th17 phenotype[33] **(Fig. 4)**. Th1 and Th17 cells are recruited back to the site of infection (the lung) and further increase the initial immune response with respectively IFN-γ and IL-17 production, macrophage activation, induction of microbicidal factors and bacterial control[25].

On the other hand, recruited monocytes not only participate actively through the production of inflammatory cytokines such as IL-1β and TNF-α[34], but also act as safeguards, exiting the bone marrow through a CCL2-dependent mechanism[35] and differentiating either into macrophages or DCs[36], further maintaining the immune response against *Mtb* **(Fig. 4)**. Within the scope of this master thesis, we intended to study 3 types of myeloid cells: AMs, monocytes and DCs. Within these 3 cells, focus was put on 2 important inflammatory axes: IL-1 and IL-12/IL-23. Further details on these cells and axes are provided in the next sections.

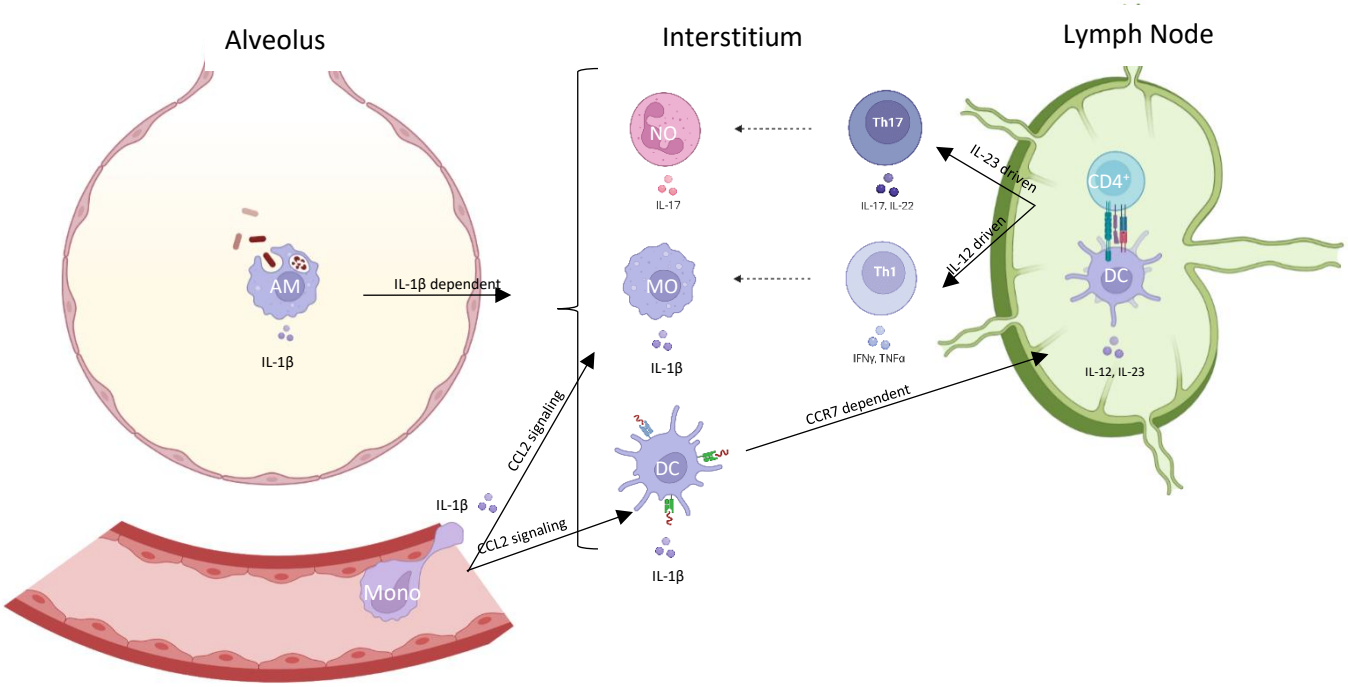

Fig. 4 Myeloid cell responses to *Mtb*. Abbreviations: AM, alveolar macrophage; CD4+, naïve T cell; DC, dendritic cell; MO, macrophage; Mono, monocyte; NO, neutrophil; Th1, T helper 1 cell ; Th17, T helper 17 cell.

Alveolar macrophages

AMs comprise a specific and distinct population of macrophages residing in the alveoli, which play determinant roles in the lung besides general immune surveillance, such as debris and surfactant clearance[37,38]. Originating from yolk sac precursors of fetal monocytes, which persist in the alveoli regardless of bone marrow contributions, these cells possess self-renewal capacities[39,40]. The development, maintenance and retention of AMs in the alveolar niche are ensured through constant supplies of granulocyte-macrophage colony stimulating factor (GM-CSF) and transforming growth factor (TGF)-β[41]. Classical surface markers of these cells include Cluster of Differentiation (CD)11c and SiglecF and the absence of CD11b, which is lost during adaptation to the lung microenvironment[42]. AMs can shift from classically-activated phenotypes (M1), induced by Th1 responses and responsible for the production of pro-inflammatory cytokines, to alternatively activated macrophages (M2), induced by Th2 responses and associated with anti-inflammatory cytokine production and apoptotic bodies engulfment[43].

Upon *Mtb* phagocytosis, AMs upregulate their IL-1β production, a process that is mediated by the ESX-1 secretion system[37], translocate to the pulmonary interstitium

and undergo proliferation, further disseminating bacilli into neutrophils and DCs[31]. Nevertheless, the contribution of AMs to *Mtb* containment is still ambiguous. Studies have highlighted Nrf2 as a regulator of the AM response, limiting their inflammatory response and making these cells an attractive niche for bacteria replication and dissemination[44–46]. In fact, infected AMs display higher mitochondrial functions and the *Mtb*'s transcriptional profile tends to be upregulated concerning fatty acids acquisition and usage[47]. Furthermore, selective depletion of AMs in mice leads to a bacterial burden decrease[47].

Monocytes

Monocytes are bone marrow-derived leukocytes which continuously circulate in the blood and display the capacity of being recruited and differentiating into either macrophages or DCs[48]. Several different monocyte subsets have been reported based on the expression of CD14 and CD16, out of which classical monocytes ($CD14^{hi}CD16^{low}$) comprise the most relevant (90% of all monocytes) human population[49]. According to provided stimuli, monocytes alternate between a pro-inflammatory state, induced during early stages of infection with phagocytic functions and production of pro-inflammatory cytokines, and an anti-inflammatory one, typically more associated with tissue repair and angiogenesis during chronic infection states[50,51].

The exact role played by monocytes during *Mtb* infection is still not yet completely understood. On the one hand, following infection, the population of classical monocytes usually decreases[48], as they are recruited to differentiate into interstitial macrophages at the site of infection, imposing a more stressful immune environment to the bacteria[52], while also producing the inflammatory mediators IL-1β, IL-6 and TNF-α[34]. Accordingly, selective depletion of interstitial macrophages in mice leads to an increase of bacterial burden[47] and prevents antigen delivery to LNs with further impact on T cell responses[53]. On the other hand, monocytes can also become infected with *Mtb*[54] and have their differentiation potential polarized into an anti-inflammatory state, becoming not only more permissive to bacterial replication and dissemination but also more prone to the production of anti-inflammatory cytokines[55].

Dendritic Cells

DCs are highly motile mononuclear phagocytes specialized in naïve T cell priming, bridging innate and adaptive immunity[56]. Phenotypically distinguishable owing to their major histocompatibility complex class II (MHCII) and CD11c expression[57], DCs are considered the most potent antigen presenting cells (APC)[58], as they can present antigens in both MHCI and MHCII formats and are the only cells able to stimulate naïve T lymphocytes[23].

While still immature, DCs express low amounts of MHCII and costimulatory molecules CD40, CD80 and CD86[59]. Upon immune injury, antigen uptake and further CCR7-dependent migration to the LN, DCs undergo a maturation process with upregulation of MHCII and the aforementioned costimulatory molecules[60].

Within *Mtb* infections, the role of DCs is somewhat ambiguous and difficult to assess, particularly owing to the functional heterogeneity of inflammatory DCs[61]. On the one hand, DCs travel to the LN and skew T cell development into a Th1 and Th17 phenotype through the production of IL-12 and IL-23, respectively[62,63]. Furthermore, they are versatile inflammatory producers of IL-1α, IL-1β, TNF-α, IL-10 and iNOS[61]. On the other hand, when infected, DCs may have their CCR7-dependent migration to the LN impaired, with further repercussions in the generation of IFN-γ and IL-17 protective responses[64,65].

The IL-1 axis in tuberculosis

The IL-1R signalling is initiated by IL-1α and IL-1β[66]. These cytokines belong to the IL-1 superfamily of ligands and receptors, exerting their biological action through the same complex of receptors: IL-1R1 primary receptor and IL-1R3 accessory chain[67,66]. IL-1α and IL-1β are transcribed upon IL-1R receptor heterodimerization, recruitment of Myd88, IRAK4 and TRAF6 intermediates and further NF-KB activation[68,69] (**Fig. 5a**).

IL-1α is produced mainly by keratinocytes, epithelial and endothelial cells[70]. Pro-IL-1α has a nuclear localization sequence within its pro-domain which, depending on environmental stimuli, allows the cytokine to shift between the nucleus and the cytosol through interactions with HAX-1[71]. Within the nucleus, given the fact that pro-IL-1α has biological activity per se, binding to chromatin and histone-modifying enzymes leads to the transcription of pro-inflammatory genes such as *il1a*, *il1b* and *il6*[67]. Upon cell injury, pro-IL-1α is released and cleaved by granzymes, further enhancing its biological activities: proliferation of B and T cells, DCs maturation, recruitment of leukocytes and angiogenesis[72] (**Fig. 5a)**.

IL-1β is mainly secreted by myeloid cells, namely monocytes, macrophages and DCs[73]. Unlike IL-1α, pro-IL-1β has no biological activity and requires further cleavage of its pro-domain to become activated[74]. This happens with the recruitment of ASC and caspase-1, which induce conformational changes within the inflammasome, subsequently leading to the cleavage of pro-IL-1β and IL-1β secretion.[70] In the myeloid compartment, IL-1β triggers APCs maturation and T cell priming into a Th17 phenotype, with subsequent stimulation of IL-17 production and neutrophil accumulation[75,67]. Within epithelial cells, IL-1β increases their adhesion molecules, facilitating immune cell migration and enhancing vascular permeability[23] **(Fig. 5a)**.

The IL-12/IL-23 axis in tuberculosis

IL-12 and IL-23 are two cytokines belonging to the IL-12 family. IL-12 is a heterodimeric cytokine composed of subunits p35 and p40, while IL-23 is composed of p19 and p40[76]. They both exert their signalling effects through Janus tyrosine kinases (JAK) and activator of transcription (STAT) modules[77]. Upon binding to their receptors, JAKs become activated, allowing phosphorylation of STATs, which dimerize, translocate to the nucleus and bind to gene promoters, regulating genetic transcription (**Fig. 5b**)[23].

On the one hand, IL-12 is mainly produced by B cells, DCs and macrophages, allowing T-bet transcription and further induction of Th1 differentiation[78]. These cells, through upregulation of an IFN-γ and TNF-α focused response, increase the recruitment of activated macrophages[79] (**Fig. 5b**). IL-12 depletion in mice compromises *Mtb* growth control and IFN-γ production[80]. Moreover, mutations in *il12rb2*, a subunit of the IL-12R, have been recently identified as genetic origins of mendelian susceptibility to mycobacterial disease, a condition caused by inborn errors of IFN-γ immunity which predisposes individuals to develop infections upon weakly virulent mycobacteria[81,82].

On the other hand, IL-23 is mainly produced by macrophages and DCs, allowing RORγt transcription and further activation of IL-17 producing cells, such as Th17, γδ T and natural killer T cells[83]. These, through upregulation of IL-17, TNF-α and GM-CSF contribute to granulocyte recruitment and activation[83] (**Fig. 5b**). Furthermore, IL-23 may act in an autocrine way, stimulating DCs and macrophages to produce IL-1β and IL-6[84]. The absence of IL-23 within *Mtb* infections impairs not only B cell follicles homeostasis, through a decrease of CXCL13 expression[85], but also Th17 responses, through a considerable loss of IL-17 mRNA expression in the lung[86].

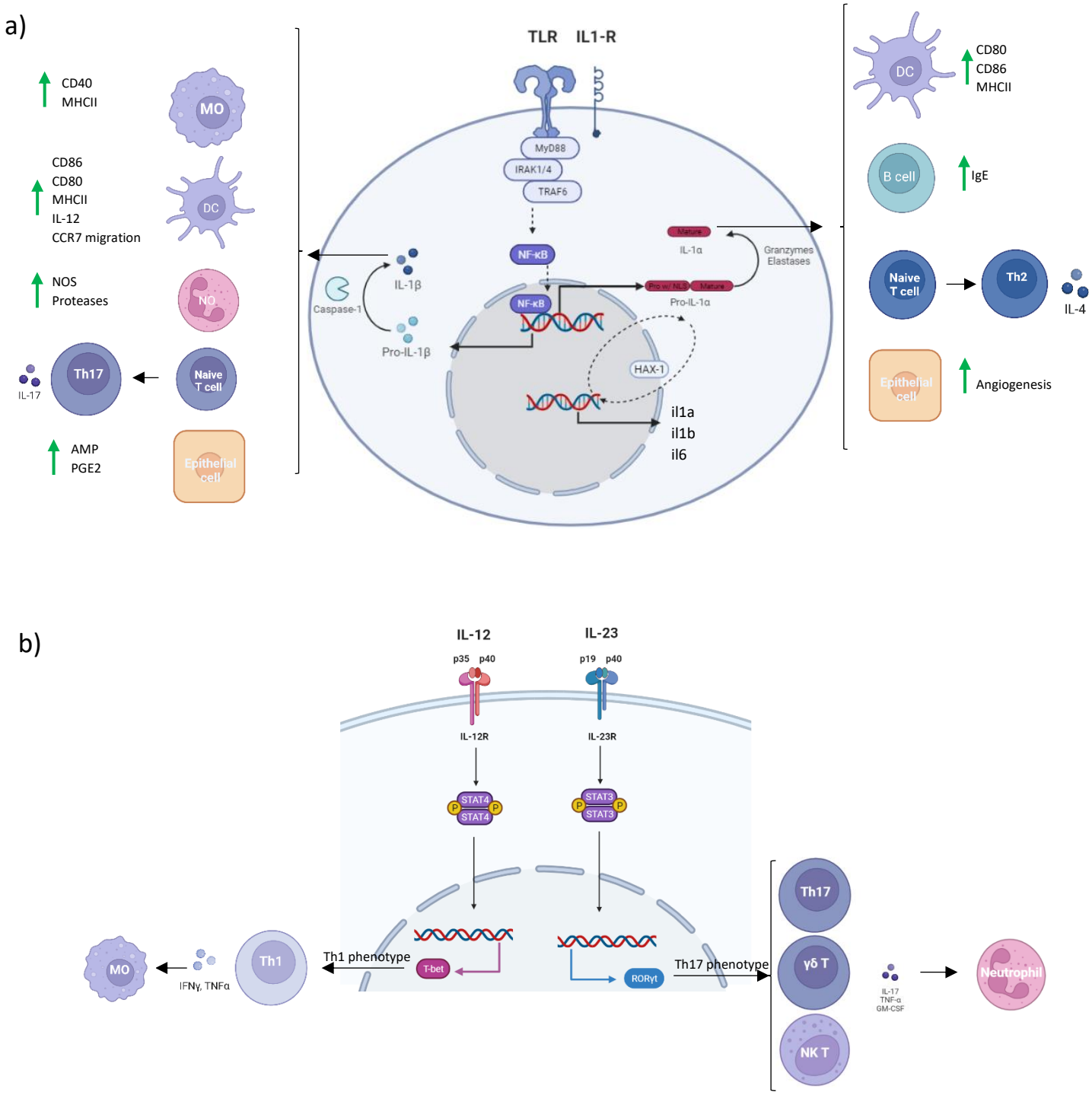

Fig. 5 IL-1 and IL-12/IL-23 axes. (a) Both pro-IL-1α and pro-IL-1β are initially transcribed through activation of the NF-KB signalling pathway. Pro-IL-1α is biologically active on its own and can shift between the cytosol and the nucleus, where it promotes transcription of *il1a*, *il1b* and *il6*. IL-1α action is augmented upon cleavage of its pro-domain, activating DCs, promoting IgE class-switch in B cells, Th2 differentiation and angiogenesis. IL-1β is cleaved after inflammasome activation, activating macrophages, DCs and neutrophils, stimulating Th17 differentiation and promoting AMPs and PGE2 production from epithelial cells. (b) IL-12 and IL-23 produce their effects through JAK-STATs signalling. IL-12 promotes transcription of T-bet and further activation of Th1 cells, which through the production of IFN-γ and TNF-α stimulate macrophages. IL-23 promotes transcription of RORγt and further activation of IL-17 producing cells, which contribute to neutrophil influx.

Mycobacterium tuberculosis genetic diversity

Mtb belongs to the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related *Mycobacterium* species comprising 9 human-adapted lineages[87]. Despite sharing 99.9% of their genome and having evolved from a common ancestor, these lineages differ significantly in their epidemiology, host range, pathogenicity and drug resistance[88,89]. As a matter of fact, random mutations involving virulent components of the cell wall and secretion system conferred each lineage with unique capacities to modulate transmissibility and disease severity[90,91].

Mtb lineages can be further divided into genetic sublineages, according to unique spoligotyping profiles and genomic signatures, which leads to specific features regarding epidemiology, virulence and immunogenicity[92]. Two *Mtb* clinical isolates, 4I2 and 6C4, under study in our group belong to the sublineage Latin-American and Mediterranean (LAM) of lineage 4. These isolates were recovered from TB patients differing in the presentation of disease severity and further shown to differentiate in their ability to induce IL-1β in macrophages[93]. While isolate 4I2 caused mild disease and induced high IL-1β levels, isolate 6C4 caused severe disease and induced low IL-1β levels[93]. This differential pathogenicity is apparently caused by bacterial genetic evolution[93], as *Mtb* 6C4 possesses two single nucleotide polymorphisms in espR, a transcriptional regulator of the ESX-1 secretion system[94], and PPE35, a protein with influence on the way macrophages process *Mtb*[95].

In vitro, *Mtb* isolate 6C4 has been shown to manipulate IL-1β production, with *Mtb* 6C4-infected BMDMs, monocytes and PBMCs exhibiting lower IL-1β secretion in comparison to isolate 4I2-infected ones[93]. Unpublished data from our group showed that in the mouse model of aerosol infection, isolate 6C4 leads to higher lung bacterial burdens (**Fig. 6a**) and pronounced neutrophil responses (**Fig. 6b, c and d**), as compared to infections with isolate 4I2. The exact reasons behind such differences have not yet been fully elucidated. Since these data are in line with patients' clinical manifestations, this model provides a platform for studying the foundations of TB severity, mainly in what IL-1 modulation is concerned. The focus of my thesis was to further elucidate these *in vivo* differences.

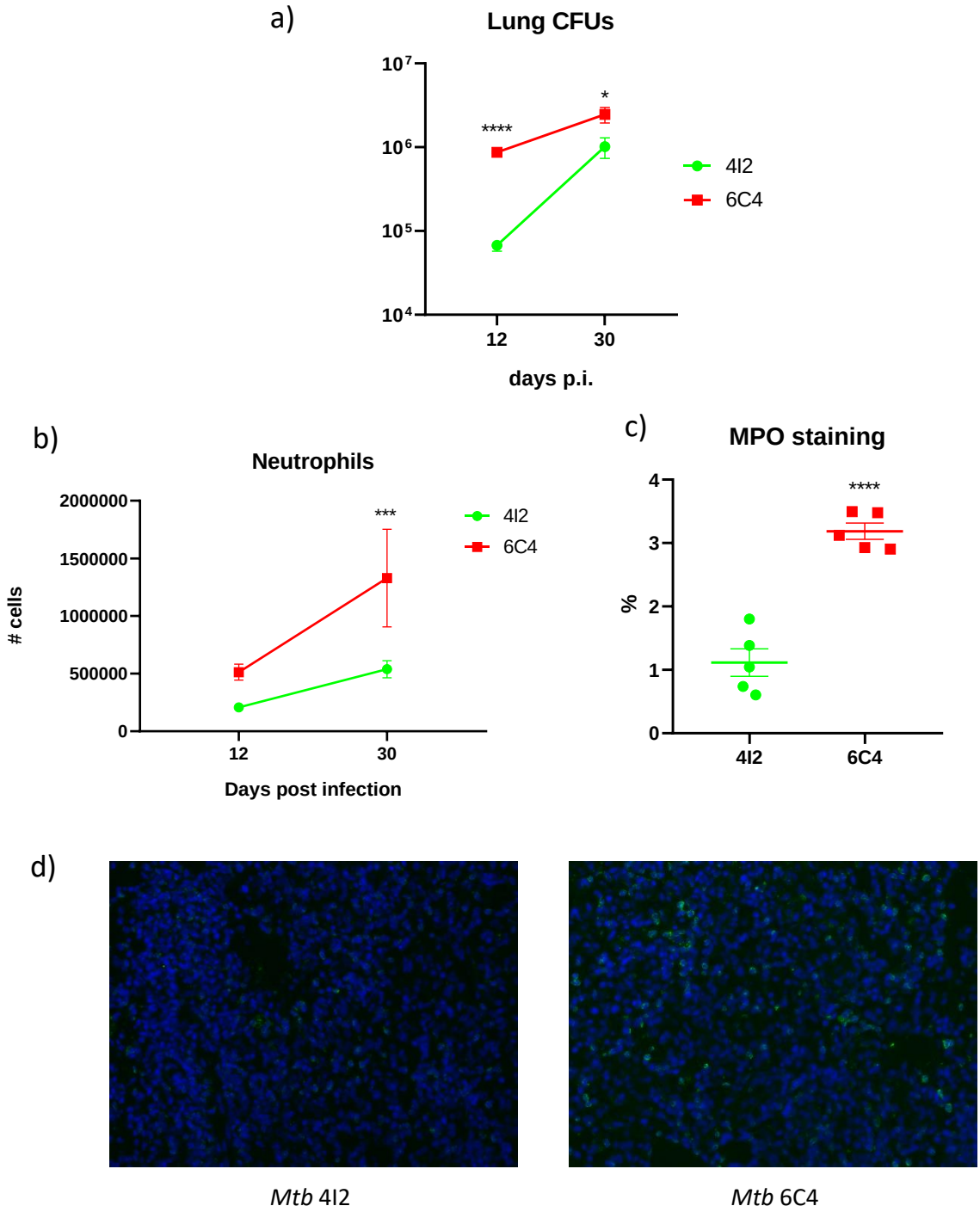

Fig. 6 *Mtb* isolate 6C4 severity is correlated to higher lung bacterial burdens and neutrophil influx. C57BL/6 mice were infected with *Mtb* isolates 4I2 and 6C4 to determine lung bacterial burden and neutrophil influx 12 and 30 days after infection. (a) Bacterial burden quantified by colony-forming-units (CFUs). (b) Neutrophil counts assessed by flow-cytometry. (c) Myeloperoxidase (MPO) quantification in immunofluorescences of *Mtb* 4I2/6C4-infected lungs, which was done by dividing the number of MPO^+ cells by the number of $DAPI^+$ ones. (d) Representative images of lung immunofluorescences for DAPI (blue) and MPO (green), at 20x magnification. Represented is mean ± SEM and each symbol refers to an individual mouse. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05, ***p<0.001 and ****p<0.0001). These data are from Silvério et al, unpublished.

Chapter 2 - Aims

Aim 1 – Understanding the regulation of IL-1 and IL-1R responses by *Mtb* 4I2 *versus* 6C4 in the myeloid compartment

We previously showed that *Mtb* isolates 4I2 and 6C4 induce differential IL-1β production in infected macrophages[93] and recently found that these two isolates are differently controlled *in vivo* (mouse) especially during early time points post-infection (**Fig. 6**). Given the importance of myeloid cell populations[61] and of IL-1β[96] in controlling *Mtb* infections *in vivo*, we started by characterizing the cell response of AMs, monocytes and DCs to these two isolates in the presence and absence of IL-1R. For this, AMs, monocytes and DCs were obtained from mice with (IL-1RfloxLyzMCre^{+}; referred to as Cre^{+}) or without (IL-1RfloxLyzMCre^{-}; referred to as Cre^{-}) IL-1R deficiency restricted to myeloid cells and infected with *Mtb* 4I2 and 6C4. The following measurements were then performed: i) bacterial replication through colony-forming-units (CFUs) assays 4 hours and 4 days after infection; ii) gene expression levels of *il10*, *tnfa*, *il6*, *ifnb*, *il1b*, *il1a*, *nos2*, *il17*, *p19*, *p35* and *p40* (6 hours after infection for AMs and monocytes and 3, 6 and 24 hours after infection for DCs) via quantitative polymerase chain reaction (qPCR); iii) protein secretion of IL-1β, TNF-α and IL-6, 24 hours after infection, with Enzyme-linked Immunosorbent Assay (ELISA) (**Fig. 7a**).

Aim 2 – Role of IL-23R during infection with *Mtb* 6C4

Considering the important role played by IL-23 in the activation of IL-17-producing cells which subsequently leads to granulocyte recruitment to the lung, we hypothesised that the enhanced neutrophil response observed in *Mtb* 6C4 infections (**Fig. 6**) may be modulated by IL-23. To dissect this hypothesis, wild-type (WT) or IL-23R deficient (IL-23R$^{-/-}$) C57BL/6 mice were aerosol-infected with *Mtb* 6C4 and the following measurements performed in the lung, 30 days after infection: i) bacterial replication via CFUs; ii) gene expression levels of *tnfa*, *il6*, *il1a*, *il1b, ifng, il18, nlrp3, il17*, *il10*, *ifnb*, *p19*, *p35* and *p40* via qPCR; iii) myeloperoxidase (MPO) quantification by immunofluorescence; iv) recruitment of immune cell populations by fluorescence-activated cell sorting (FACS) (**Fig. 7b**).

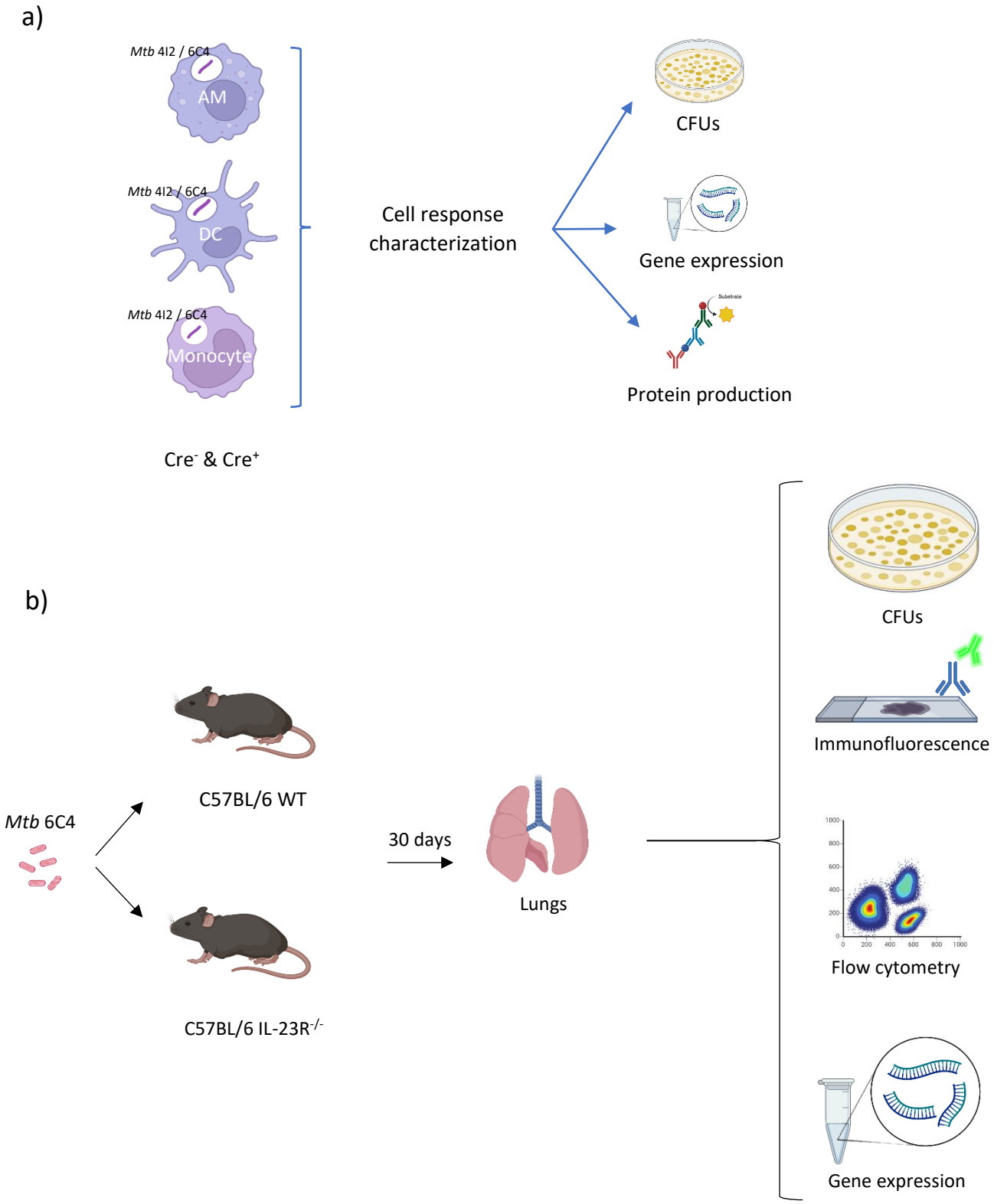

Fig. 7 Master thesis aims. (a) Schematic representation of Aim 1. (b) Schematic representation of Aim 2.

Chapter 3 – Results

Modulation of the alveolar macrophage response by *Mtb* isolates 4I2 and 6C4

Protocol optimisation

AMs, being located in the alveoli, can be isolated from the bronchoalveolar lavage (BAL) fluid of mice, which is obtained through several washes of lung airways with a saline solution, in this case BAL buffer (PBS + EDTA + FBS). Within this step, several parameters are critical for extracting as many cells as possible: quickness of the harvesting, temperature of the buffer and number of used aliquots in the lavage[97]. Handling animals immediately after sacrifice, using 37°C pre-warmed BAL buffer and 9 flushes per lavage allowed the extraction of approximately 2*10^5 cells/animal. These were then plated in 96-well plates and rested for 24 hours before proceeding with the infection procedure at a multiplicity of infection (MOI) of 2 (**Fig. 8**).

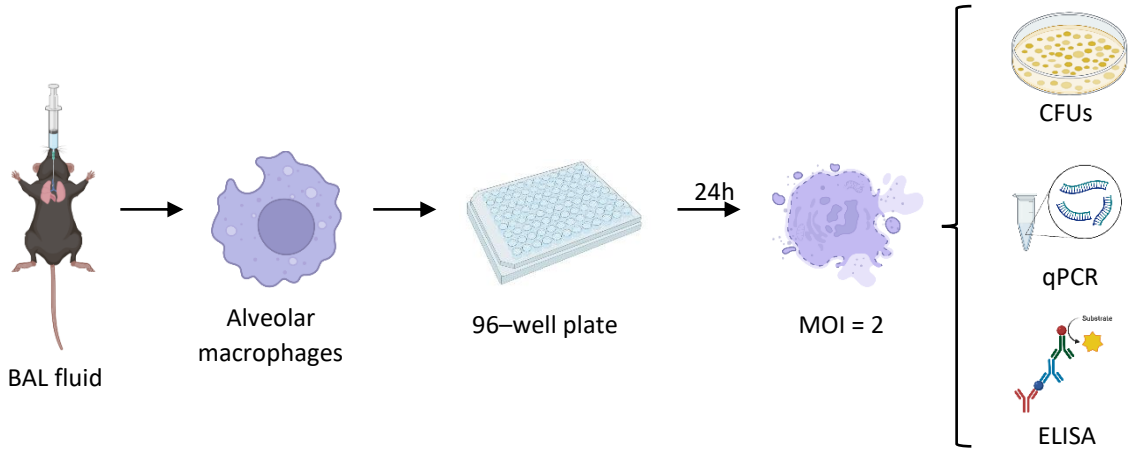

Fig. 8 Schematic representation of the protocol used to study the response of AMs to infection by *Mtb* isolates 4I2 and 6C4. AMs were isolated from the BAL fluid of mice, plated in 96-well plates and left to rest for 24 hours before infection with 4I2 and 6C4 *Mtb* isolates at a MOI of 2. CFUs were assessed both 4 hours and 4 days after infection, gene expression levels at 6 hours post-infection by qPCR and cytokine expression 24 hours after infection by ELISA.

Alveolar macrophages are permissive to *Mtb*

We started by comparing the response of WT AMs to each *Mtb* isolate under study. Concerning bacterial control, we observed that intracellular CFU numbers were higher upon infection with *Mtb* 4I2, both initially and 4 days after infection, as compared to *Mtb* 6C4 (**Fig. 9a**). Taking fold change into consideration, we observed that bacterial burden change over the 4 days of the experiment was similar between isolates 4I2 and 6C4 (**Fig. 9b**), suggesting a similar control of both *Mtb* isolates by AMs.

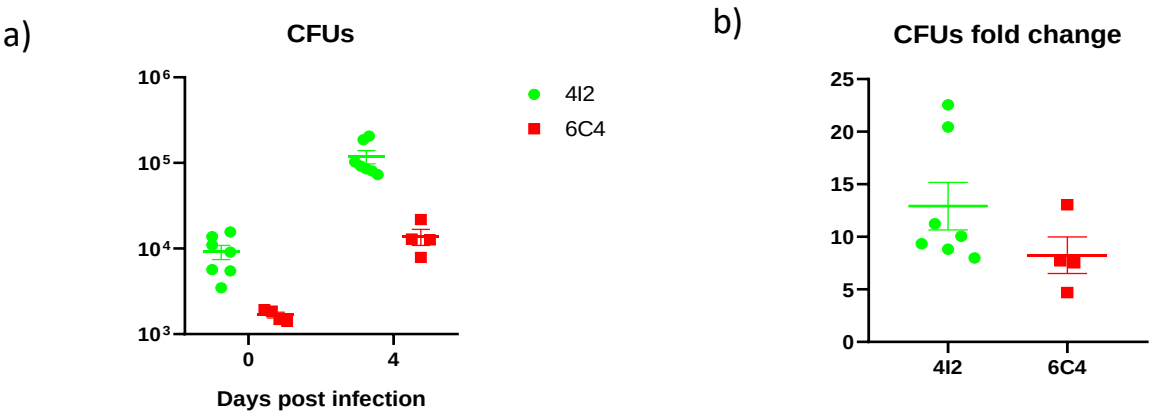

Fig. 9 Alveolar macrophages restrict *Mtb* 4I2 and 6C4 growth to the same extent. Number (a) and fold change (b) of intracellular CFUs in AMs infected with 4I2 and 6C4 *Mtb* isolates at a MOI of 2. CFU numbers were determined at 4 hours and 4 days after infection. Fold change was calculated by dividing each CFU value at day 4 by the mean of day 0. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test.

Transcriptional alterations induced upon infection of AMs were assessed by qPCR at 6 hours post-infection. Overall, the expression of the analysed genes was higher for *Mtb* 6C4-infected AMs, with statistically significant differences for the inflammatory mediators *il6*, *il1b* and *il17* (**Fig. 10a**). This stronger transcriptional activation of AMs by *Mtb* 6C4 was however not reflected in terms of protein secretion. While IL-1β and IL-6 were produced to the same extent by AMs infected with *Mtb* 4I2 or 6C4, TNF-α secretion was increased upon infections with isolate 6C4 (**Fig. 10b**).

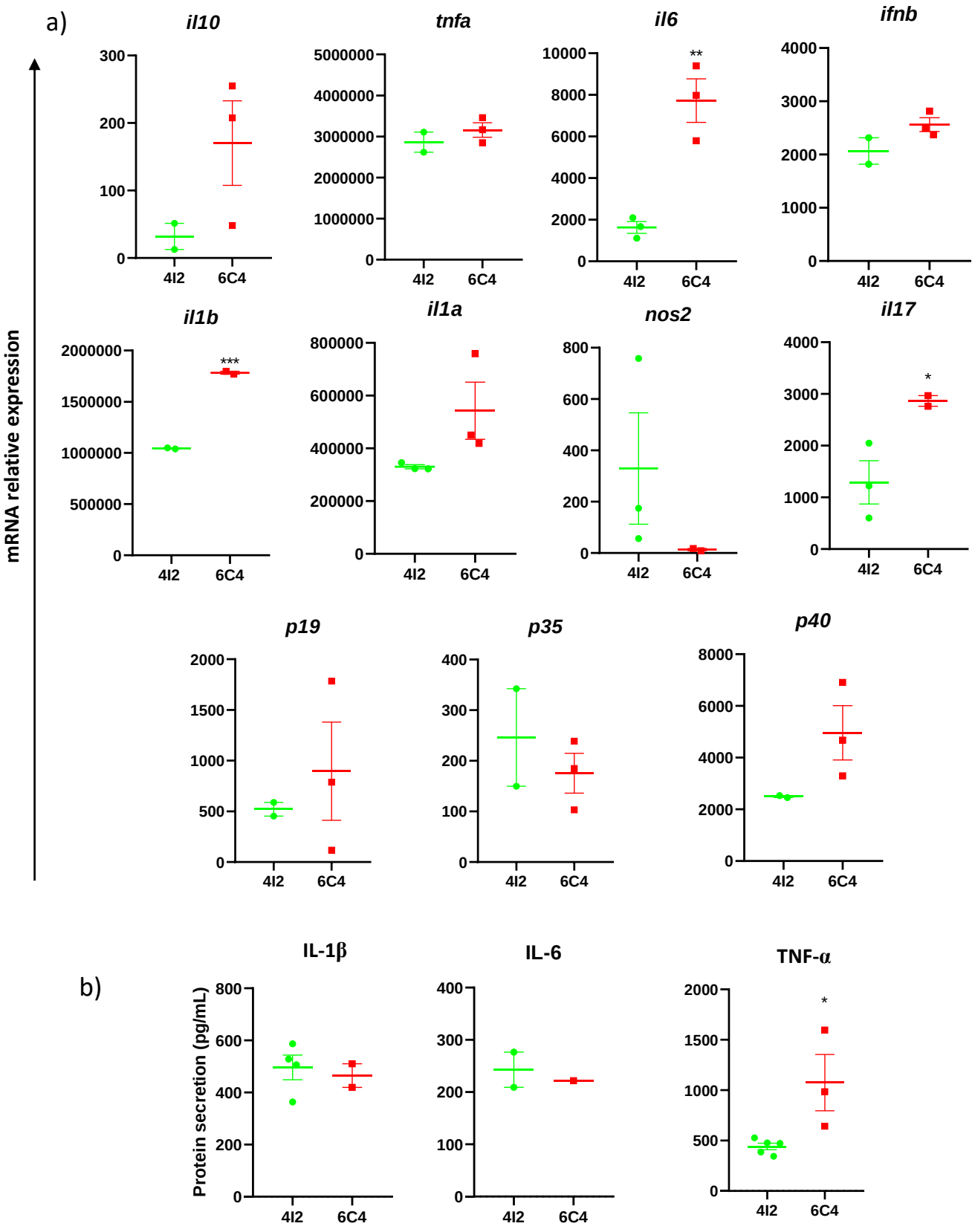

Fig. 10 Alveolar macrophages upregulate inflammatory genes upon *Mtb* 6C4 infections. AMs were generated and infected with 4I2 and 6C4 clinical isolates. (a) Six hours after infection, cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. 24 hours after infection, cell supernatants were collected and the protein levels of the indicated cytokines were measured through ELISA. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05, **p<0.01 and ***p<0.001).

Alveolar macrophages rely on IL-1R signalling to produce inflammatory mediators, but not for *Mtb* control

Next, we aimed at investigating whether IL-1R signalling impacted the *in vitro* response of AMs to *Mtb* isolates 4I2 and 6C4. For that purpose, we used a mouse model where the IL-1R has been specifically deleted from the myeloid cell compartment using the Cre/flox technology. Firstly, we validated the IL-1R knockdown in AMs from Cre^+ mice. Accordingly, *Il1r* expression levels were assessed in non-infected Cre^- and Cre^+ AMs, yielding a clear difference between genotypes (**Fig. 11**).

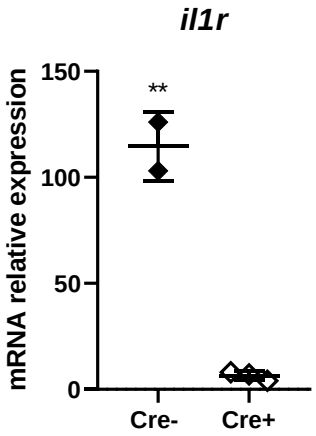

Fig. 11 IL-1R knockdown validation in alveolar macrophages. Cell cultures of IL-1RfloxLyzMCre$^-$ and Cre$^+$ AMs were lysed, their RNA extracted and converted to cDNA, after which *il1r* expression levels were assessed with qPCR. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (**p<0.01).

We then assessed the impact of IL-1R deletion on bacterial control. Due to the limited amount of harvested AMs, experiments assessing bacterial growth could only be performed for one *Mtb* isolate. We opted for isolate 4I2, given previous experiments from our group displaying higher differences in lung CFUs for *Mtb* 4I2-infected Cre^+ mice in comparison to *Mtb* 6C4-infected ones (unpublished data). Regarding bacterial loads, the number of CFUs between Cre^- and Cre^+ AMs infected with *Mtb* 4I2 was levelled (**Fig. 12a**) and so was their fold change (**Fig. 12b**), suggesting IL-1R signalling is dispensable within AMs for *Mtb* 4I2 growth control.

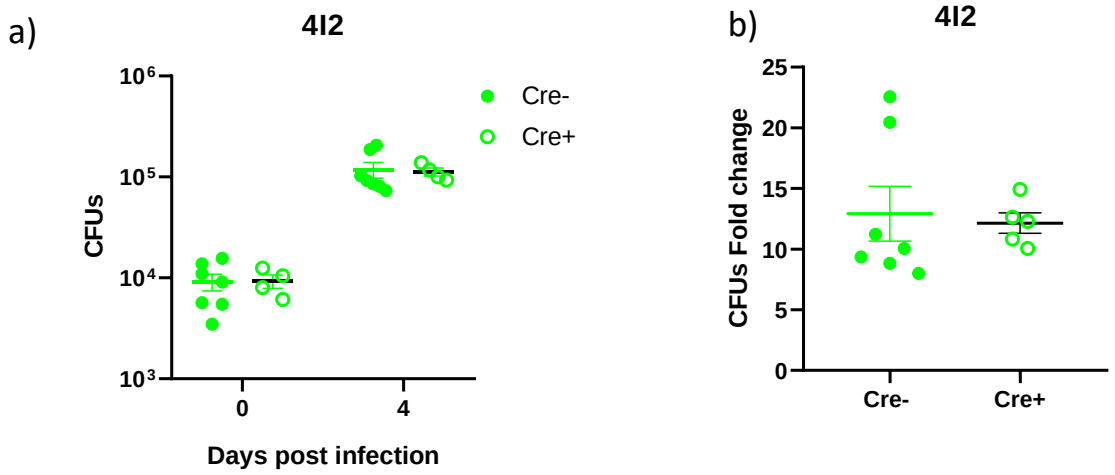

Fig. 12 *Mtb* 4I2 growth within alveolar macrophages is IL-1 independent. IL-1RfloxLyzMCre$^-$ and Cre$^+$ AMs were generated and infected with *Mtb* 4I2 at a MOI of 2. (a) Number of intracellular CFUs at 4 hours and 4 days post-infection. (b) CFUs fold change assessment was calculated by dividing each CFU value at day 4 by CFUs mean at day 0. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test.

As for gene expression levels, we observed a generic mRNA upregulation in AMs of the Cre$^+$ group, with statistically significant differences for *ifnb* and *il6* upon *Mtb* 6C4 infections (**Fig. 13a**). Furthermore, considering gene expression levels between *Mtb* isolates in Cre$^+$ AMs, isolate 6C4 led to an overall higher enhancement of mRNA in comparison with isolate 4I2. Nonetheless, this mRNA tendency was not completely translated into protein production. On the one hand, IL-1β and IL-6 proteins were enhanced in *Mtb* 6C4-infected Cre$^+$ cells. On the other hand, upon infections with *Mtb* 4I2 and 6C4, lack of IL-1R signalling led to a decrease of TNF-α secretion (**Fig. 13b**).

Altogether, despite having no influence on bacterial control, IL-1R signalling appears to be relevant in AMs for maintaining balanced levels of inflammatory responses.

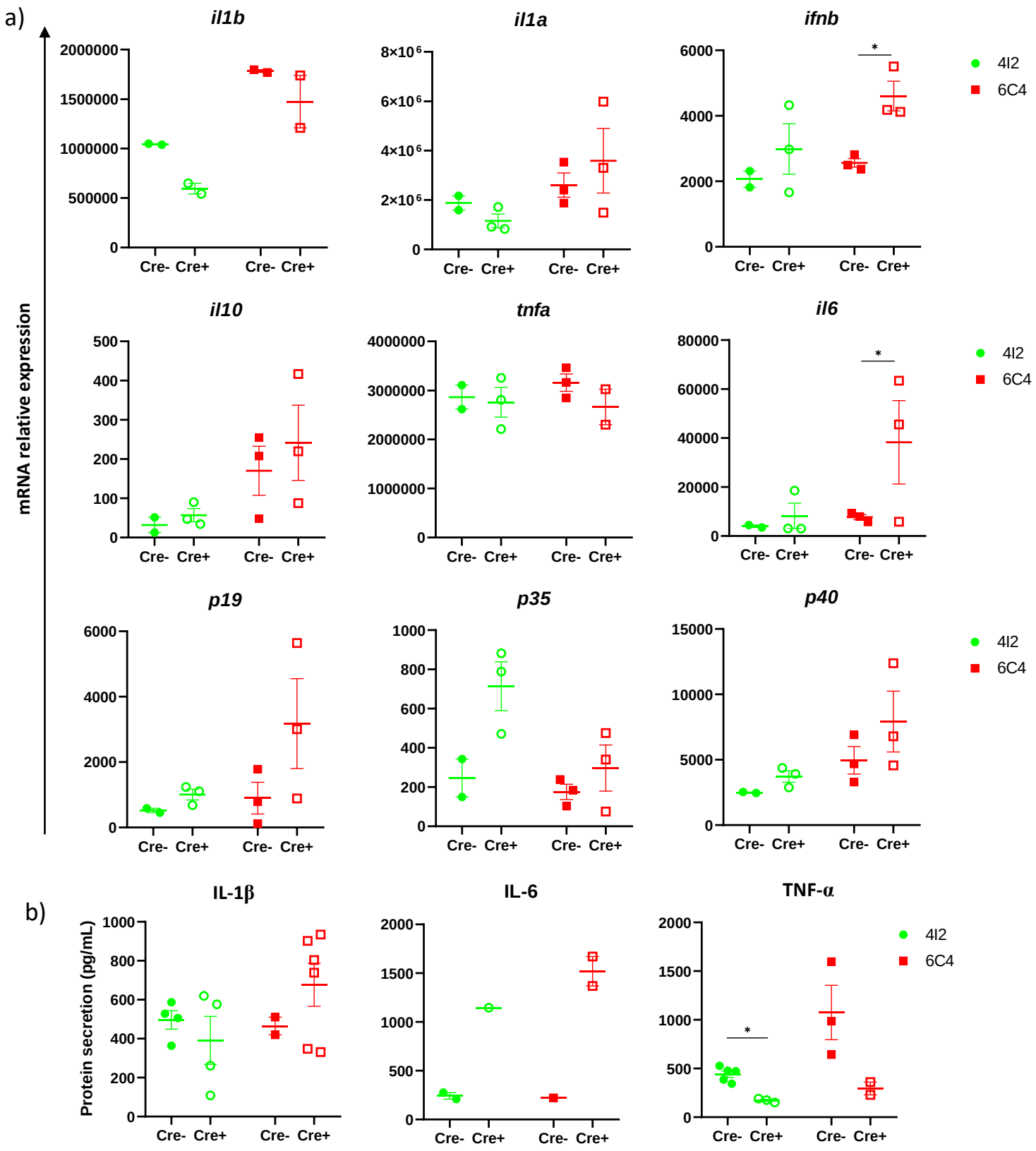

Fig. 13 IL-1R abrogation dysregulates gene expression and protein secretion in alveolar macrophages. IL-1RfloxLyzMCre⁻ and Cre⁺ AMs were generated and infected with 4I2 and 6C4 clinical isolates. (a) Six hours after infection, cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. (b) 24 hours after infection, cell supernatants were collected and the protein levels of the indicated cytokines were measured through ELISA. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Modulation of the monocyte response by *Mtb* isolates 4I2 and 6C4

Protocol optimisation

Considering monocytes derive from the bone marrow, the first step within their isolation protocol was harvesting mouse bone-marrow cells from the tibia and femur. Monocytes were further isolated from the obtained cell suspensions by magnetic separation and a negative selection cocktail[98], which labels undesired bone marrow immune cells with antibodies and magnetic particles. These can be discarded, leaving behind the monocyte fraction, which was then plated in 24-well plates at a density of 5*10^5 cells/mL. After a 24h rest, infection procedures at a MOI of 2 were performed (**Fig. 14**).

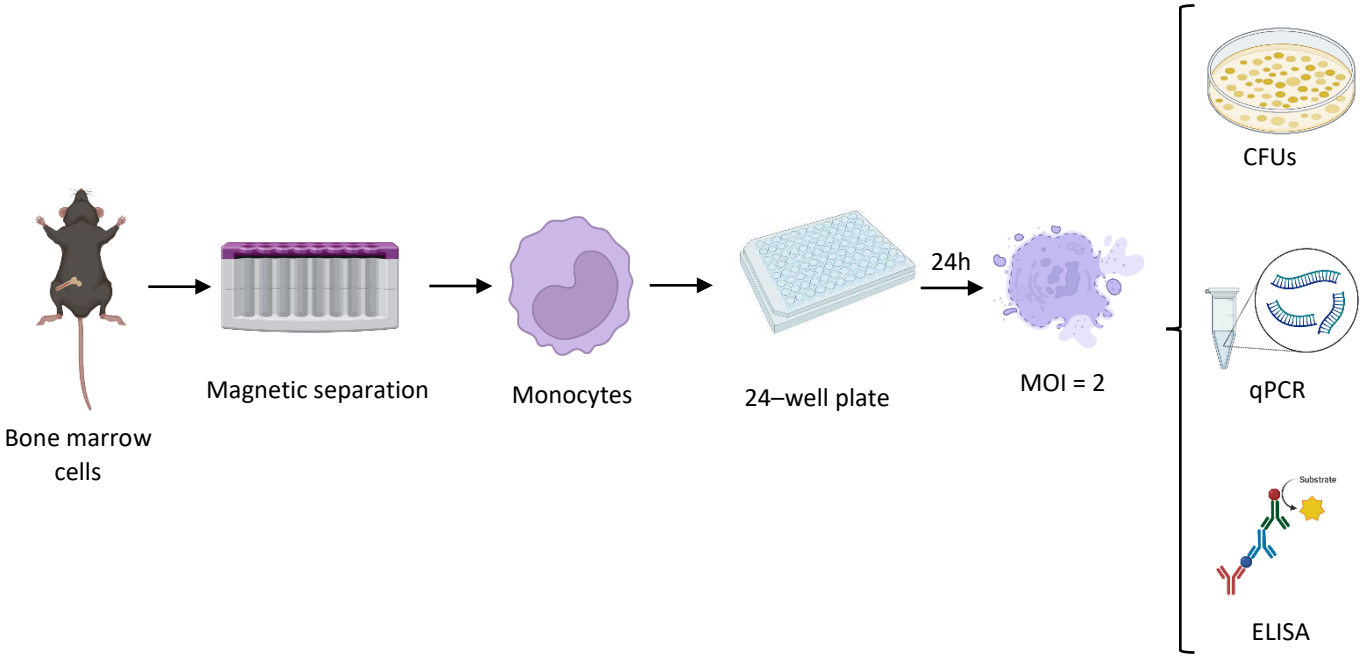

Fig. 14 Schematic representation of the protocol used to study the response of monocytes to *Mtb* 4I2 and 6C4 isolates. Monocytes were isolated from bone marrow cells, via magnetic separation and negative selection with a commercial kit, after which cells were immediately plated in 24-well plates and left to rest for 24 hours before infection with *Mtb* isolates 4I2 and 6C4 at a MOI of 2. CFUs were assessed both 4 hours and 4 days after infection, gene expression levels at 6 hours post-infection by qPCR and cytokine expression 24 hours after infection by ELISA.

Monocytes are permissive to *Mtb* replication

Our first approach was to measure the response of WT monocytes to both isolates 4I2 and 6C4. Regarding bacterial loads, intracellular CFU numbers either 4 hours or 4 days after infection were equivalent between *Mtb* isolates (**Fig. 15a**). CFUs fold change, although similar between isolates 4I2 and 6C4, revealed a considerable extent of bacterial replication within monocytes, with *Mtb* colonies increasing approximately 20x (**Fig. 15b**). This suggests that, in this *in vitro* system, monocytes were less able to control bacterial growth as compared to AMs and may, thus, be preferential targets for *Mtb* replication.

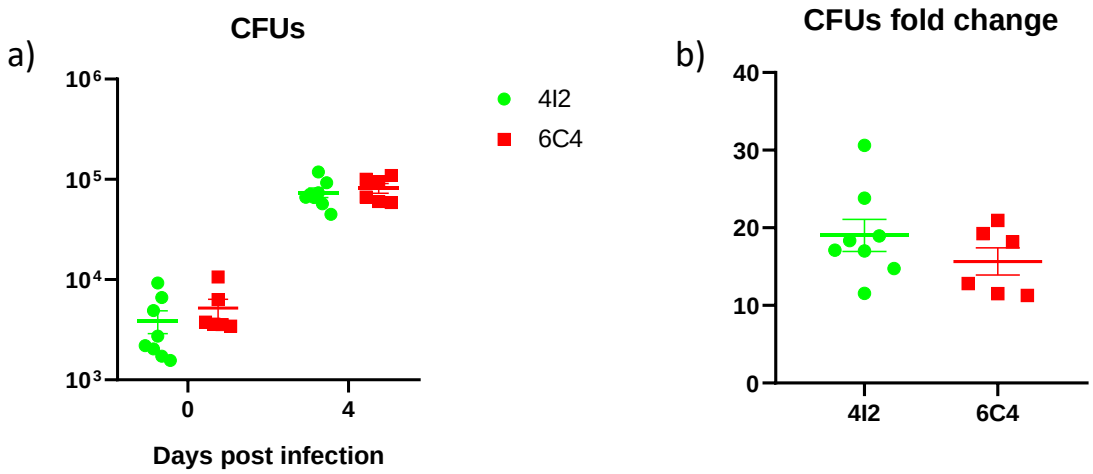

Fig. 15 Monocytes are equally permissive to *Mtb* 4I2 and 6C4 replication. Intracellular CFU numbers (a) and fold change (b) in monocytes infected with *Mtb* 4I2 and 6C4 at a MOI of 2. CFU numbers were determined at 4 hours and 4 days after infection and fold change assessment was calculated after 4 days of infection in comparison to CFUs mean at day 0. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test.

Considering the transcriptional analysis of the assessed genes, we found that the expression of the majority of mRNA was more pronounced in monocytes infected with *Mtb* 6C4 as compared to those of *Mtb* 4I2, exhibiting statistically significant differences for *il1b*, *il1a*, *il6*, *il10*, *tnfa*, *p35* and *p40*. Following the exact opposite trend, *ifnb* genetic levels were almost absent upon isolate 6C4 infections (**Fig. 16a**). Regarding protein secretion, TNF-α and IL-6 translation levels were correlated with the aforementioned gene expression augmentation (**Fig. 16b**). We did not detect IL-1β protein in monocytes,

independently of the *Mtb* isolate used during infection. This finding will require further investigation.

Nevertheless, this ability to upregulate inflammatory mediators, particularly against *Mtb* 6C4, sharply contrasts with monocytes high bacterial burden, suggesting an important but still limited role for these cells within *Mtb* infections.

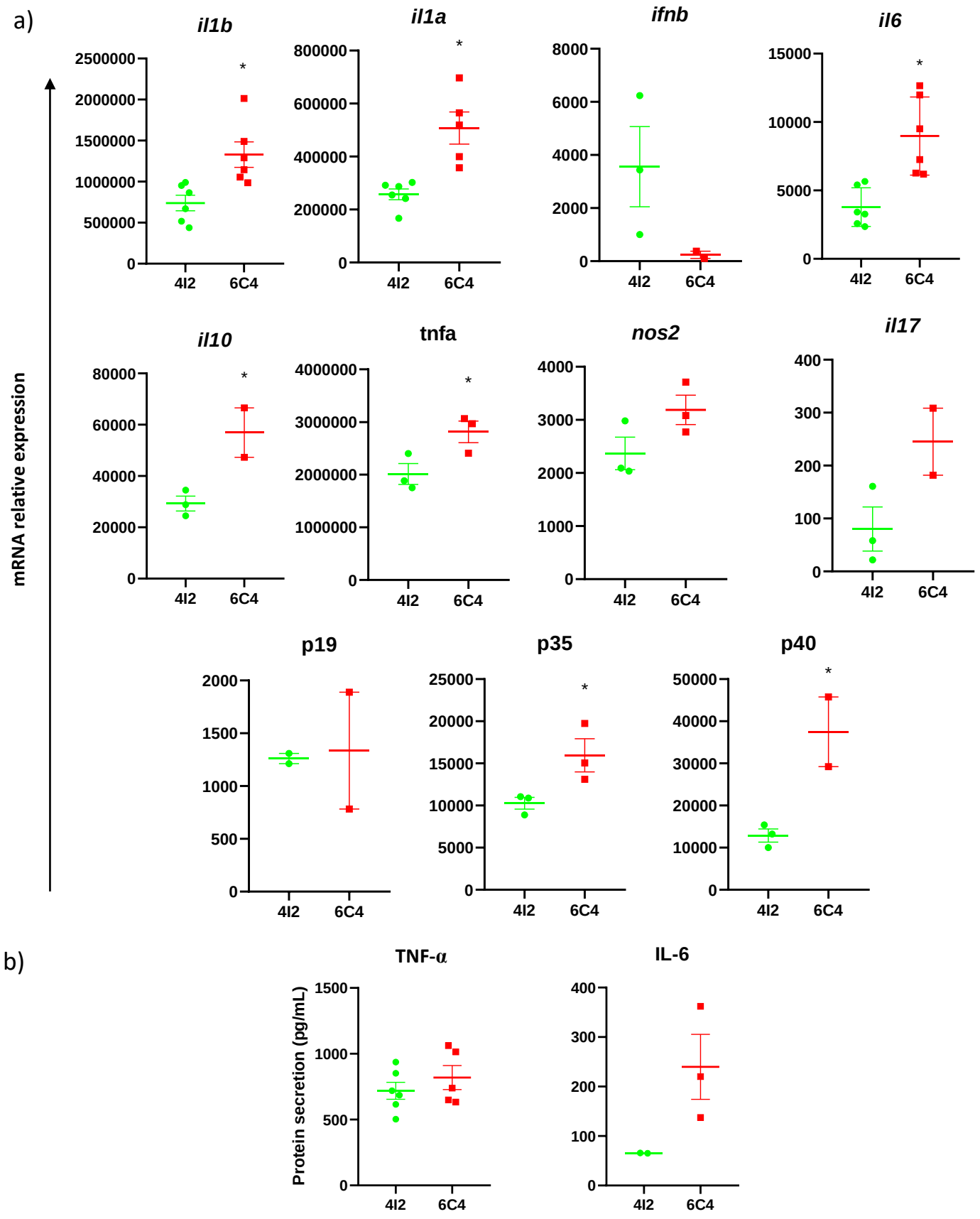

Fig. 16 *Mtb* 6C4-infected monocytes upregulate inflammatory markers. Monocytes were generated and infected with 4I2 and 6C4 clinical isolates. (a) Six hours after infection, cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. (b) 24 hours after infection, cell supernatants were collected and the protein levels of the indicated cytokines were measured through ELISA. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Monocytes rely on IL-1R signalling to mediate their genetic inflammatory network

We then proposed to investigate whether IL-1R signalling had any impact on the *in vitro* response of monocytes to each *Mtb* isolate, using the same mouse model as previously, where IL-1R is specifically deleted in the myeloid compartment recurring to the Cre/flox technology. To confirm the expected IL-1R abrogation in the Cre^+ genotype, *il1r* expression levels were assessed in both non-infected Cre^- and Cre^+ cells. Despite both genotypes showing a relatively high mRNA expression, there were statistically significant differences between them (**Fig. 17**).

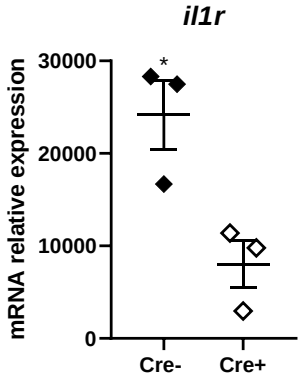

Fig. 17 IL-1R knockdown validation in monocytes. Cell cultures of IL-1RfloxLyzMCre$^-$ and Cre$^+$ monocytes were lysed, their RNA extracted and converted to cDNA, after which *il1r* expression levels were assessed with qPCR. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Regarding the transcription of the evaluated genes, we found that abrogation of IL-1R in monocytes impacted the transcription of some, but not all genes. On the one hand, *il1b* was upregulated in Cre^+ monocytes, regardless of the infecting *Mtb* isolate. On the other hand, in the Cre^+ group *il10* was upregulated in *Mtb* 4I2 infections while *ifnb* was enhanced upon isolate 6C4 infections (**Fig. 18**). These results suggest the importance of IL-1R signalling in the regulation of the expression of some, but not all monocytes inflammatory markers.

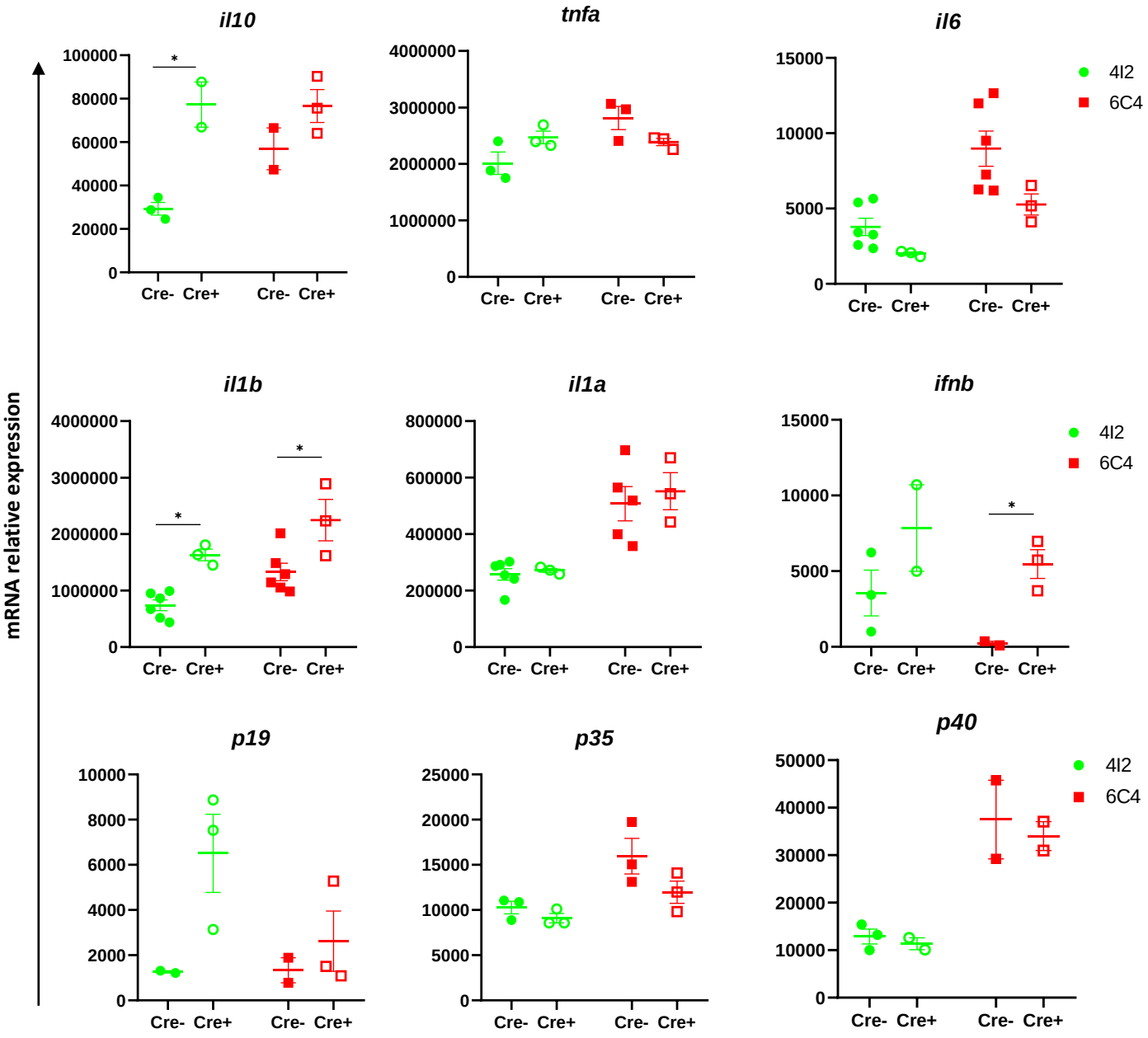

Fig. 18 Monocytes require IL-1R signalling to balance their inflammatory genetic network. IL-1RfloxLyzMCre^{-} and Cre^{+} monocytes were generated and infected with isolates 4I2 and 6C4. Six hours after infection, cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Modulation of dendritic cell responses by *Mtb* 4I2 and 6C4

Protocol optimisation

The first step in obtaining cultures of DCs was to optimise their harvesting protocol and demonstrate that they were functionally active upon culture. DCs, just as monocytes, are initially derived from bone marrow cells but require several GM-CSF supplementations during at least 9 days to attain complete differentiation (**Fig. 19a**). Within 9 days of culture in GM-CSF supplemented medium and subsequent 24 hours of rest, approximately 70% of the plated cells were $CD11c^+$, as assessed by FACS, and thus differentiated into DCs (**Fig. 19b**). With this purity value, in every subsequent experiment, DCs were always plated at day 9 and left to rest for 24 hours before being infected at a MOI of 2 to analyse CFUs, gene expression and protein production.

The expression of MHCII and B7 (CD80 and CD86) costimulatory molecules, as correlators of inflammatory activation[99], was quantified upon stimulation of DCs with bacterial lipopolysaccharide (LPS). The frequency of $MHCII^+CD86^+$ and $MHCII^+CD80^+$ cells increased by around 30% after stimulation, demonstrating that cultured DCs were functionally responding to inflammatory stimuli (**Fig. 19c**).

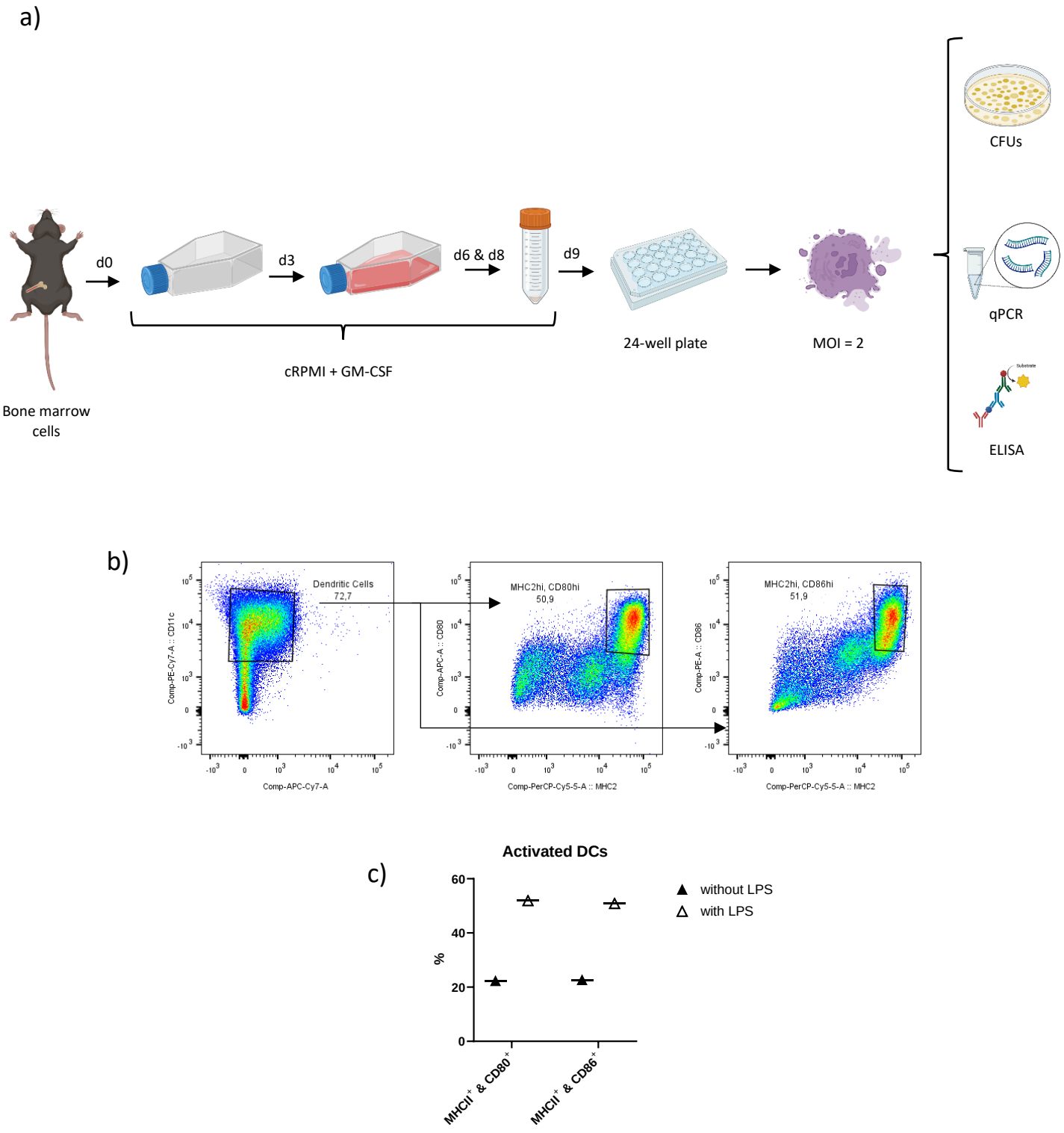

Fig. 19 Schematic representation of the protocol used to study dendritic cells response to *Mtb* isolates 4I2 and 6C4. (a) DCs were differentiated from harvested bone marrow cells during 9 days with several resuspensions and supplementations with GM-CSF. Cells were plated in 24-well plates and left to rest for 24 hours before infection with isolates 4I2 and 6C4 at a MOI of 2. (b) Gating strategy for DCs was based on CD11c and activated DCs were further distinguished according to their MHCII, CD80 and CD86 expression. (c) Comparison of the percentage of activated DCs with and without LPS stimulation.

Dendritic cells control *Mtb* growth and are effective producers of inflammatory mediators

We started by analysing the response of WT DCs to *Mtb* isolates 4I2 and 6C4. Assessing bacterial burden, both the number of CFUs after 4 days of infection (**Fig. 20a**) and respective fold change (**Fig. 20b**) were higher for DCs infected with *Mtb* 4I2. Nevertheless, it is interesting to note the lower CFUs fold change in DCs as compared to AMs and monocytes, which suggests that these cells may be equipped with more effective intracellular bactericidal mechanisms.

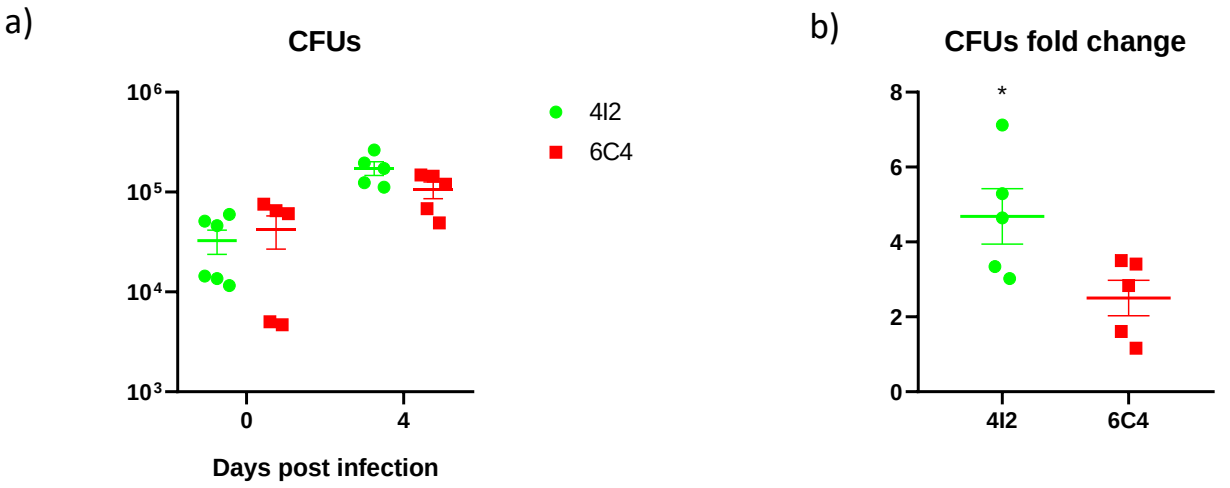

Fig. 20 Dendritic cells are more permissive to *Mtb* 4I2. Number and fold change of intracellular CFUs in DCs infected with *Mtb* 4I2 and 6C4 at a MOI of 2. CFU numbers (a) were determined at 4 hours and 4 days after infection and fold change (b) was calculated after 4 days of infection in comparison to CFUs mean at day 0. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Regarding the evaluated mRNA, DCs infected with isolate 6C4 enhanced their overall inflammatory gene expression levels more than those infected with *Mtb* isolate 4I2, with statistically significant differences for *il1b* and *il1a* (**Fig. 21a**). On the other hand, *il10*, *tnfa*, *ifnb* and *p19* did not show a continuous kinetic evolution, exhibiting a sharp increase after 3 hours of infection followed by a stabilization of their expression levels. Concerning protein secretion, IL-1β was increased for *Mtb* 4I2-infected DCs, demonstrating a clear opposite trend to its genetic levels, whereas TNF-α protein production was similar between *Mtb* isolates. IL-6 was the only cytokine with a

concomitant enhancement of its gene and protein levels upon *Mtb* 6C4 infections (**Fig. 21b**).

All in all, among the assessed myeloid cells, DCs exhibited the highest production of inflammatory mediators and best levels of bacterial control, suggesting an important and versatile role for these cells within *Mtb* infections.

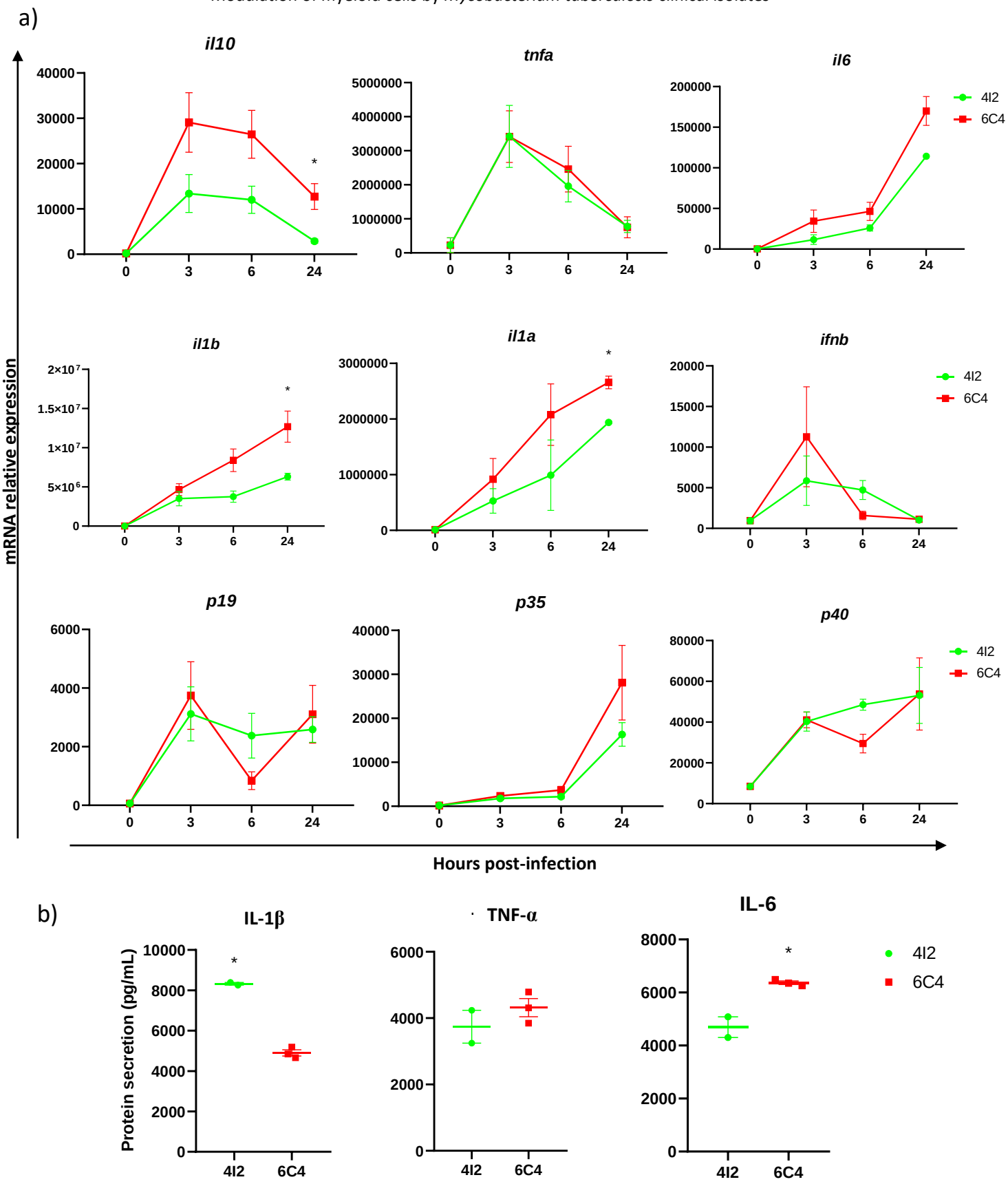

Fig. 21 Dendritic cells upregulate gene expression levels upon *Mtb* 6C4 infections. DCs were generated and infected with 4I2 and 6C4 clinical isolates. (a) 3, 6 and 24 hours after infection cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. (b) 24 hours after infection, cell supernatants were collected and the protein levels of the indicated cytokines were measured through ELISA. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Dendritic Cells rely on IL-1R signalling for bacterial control and cytokine gene transcription

Aiming to depict the influence of the IL-1R signalling in the *in vitro* response of DCs, we recurred to the previously used mouse model, where IL-1R is specifically deleted in the myeloid compartment with the Cre/flox technology. To make sure of the effectiveness of IL-1R abrogation in Cre^+ DCs, *il1r* gene expression levels were assessed in non-infected Cre^- and Cre^+ cells. As expected, a clear difference was observed between genotypes, validating the efficacy of the Cre^+ system in DCs (**Fig. 22**).

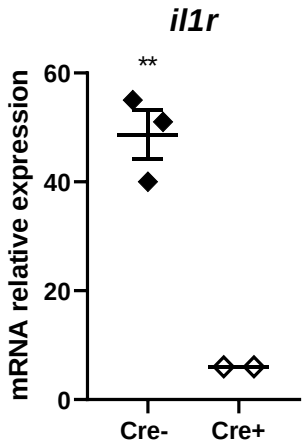

Fig. 22 IL-1R knockdown validation in dendritic cells. Cell cultures of IL-1RfloxLyzMCre$^-$ and Cre$^+$ DCs were lysed, their RNA extracted and converted to cDNA, after which *il1r* expression levels were assessed with qPCR. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (**p<0.01).

Considering bacterial growth after 4 days of infection, the number of intracellular CFUs of both isolates 4I2 (**Fig. 23a**) and 6C4 (**Fig. 23b**) was approximately the same within Cre^- and Cre^+ DCs. Nonetheless, when fold change is taken into account, a significant increase of *Mtb* 4I2 and 6C4 burden in Cre^+ DCs was observed (**Fig. 23c**), suggesting that DCs depend on IL-1R signalling for the optimal control of *Mtb*. Given a certain degree of variability obtained for the Cre^- genotype on day 0 of infection (**Fig. 23a and b**), this experiment will have to be repeated to reinforce this conclusion.

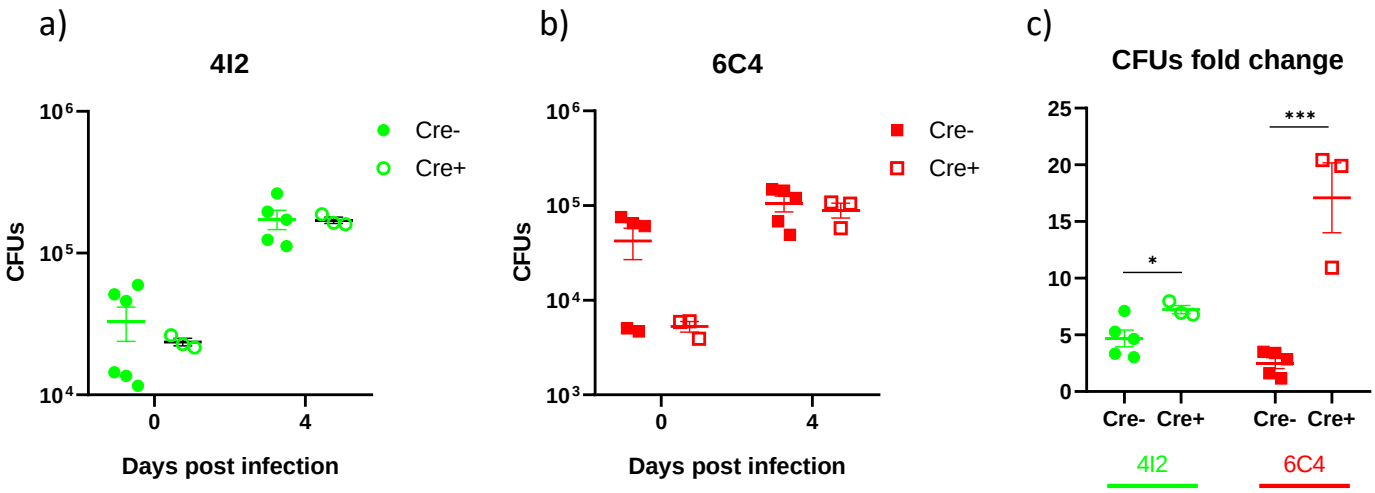

Fig. 23 Dendritic cells require IL-1R signalling to contain *Mtb* 4I2 and 6C4 replication. Number (a and b) and fold change (c) of CFUs from IL-1RfloxLyzMCre$^-$ and Cre$^+$ DCs infected with 4I2 and 6C4 *Mtb* isolates at a MOI of 2. CFU numbers were determined at 4 hours and 4 days after infection. Fold change was calculated after 4 days of infection in comparison to CFUs mean at day 0. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05 and ***p<0.001).

Regarding evaluated genes in infections with *Mtb* isolate 4I2, a decrease in mRNA expression levels after 24 hours of infection was observed for every assessed cytokine in Cre$^+$ DCs, with statistically significant differences for *il10*, *tnfa*, *il6*, *il1b*, *p19* and *p35* (**Fig. 24a**). Once again, *il10*, *tnfa*, *ifnb* and *p19* did not demonstrate a continuous kinetic evolution, but instead a sharp increase at 3 hours post-infection, followed by a stabilization of their expression levels. This decreasing mRNA tendency was not reflected in protein levels, with the secretion of IL-1β, TNF-α and IL-6 being levelled between Cre$^-$ and Cre$^+$ DCs (**Fig. 24b**).

Regarding the assessed panel of genes in infections with *Mtb* 6C4, mRNA expression levels were unbalanced between Cre$^-$ and Cre$^+$ cells (**Fig. 25a**), with *il1b* demonstrating a statistically significant decrease after 24 hours of infection. *Il10*, *tnfa*, *ifnb* and *p19* maintained a kinetic evolution only until 6 hours post-infection. Protein secretion was similar between both genotypes (**Fig. 25b**).

These results suggest not only that DCs are dependent on IL-1R signalling to upregulate inflammatory markers in *Mtb* infections but also that these cells possess feedback mechanisms assuring protein secretion upon IL-1R deficiency.

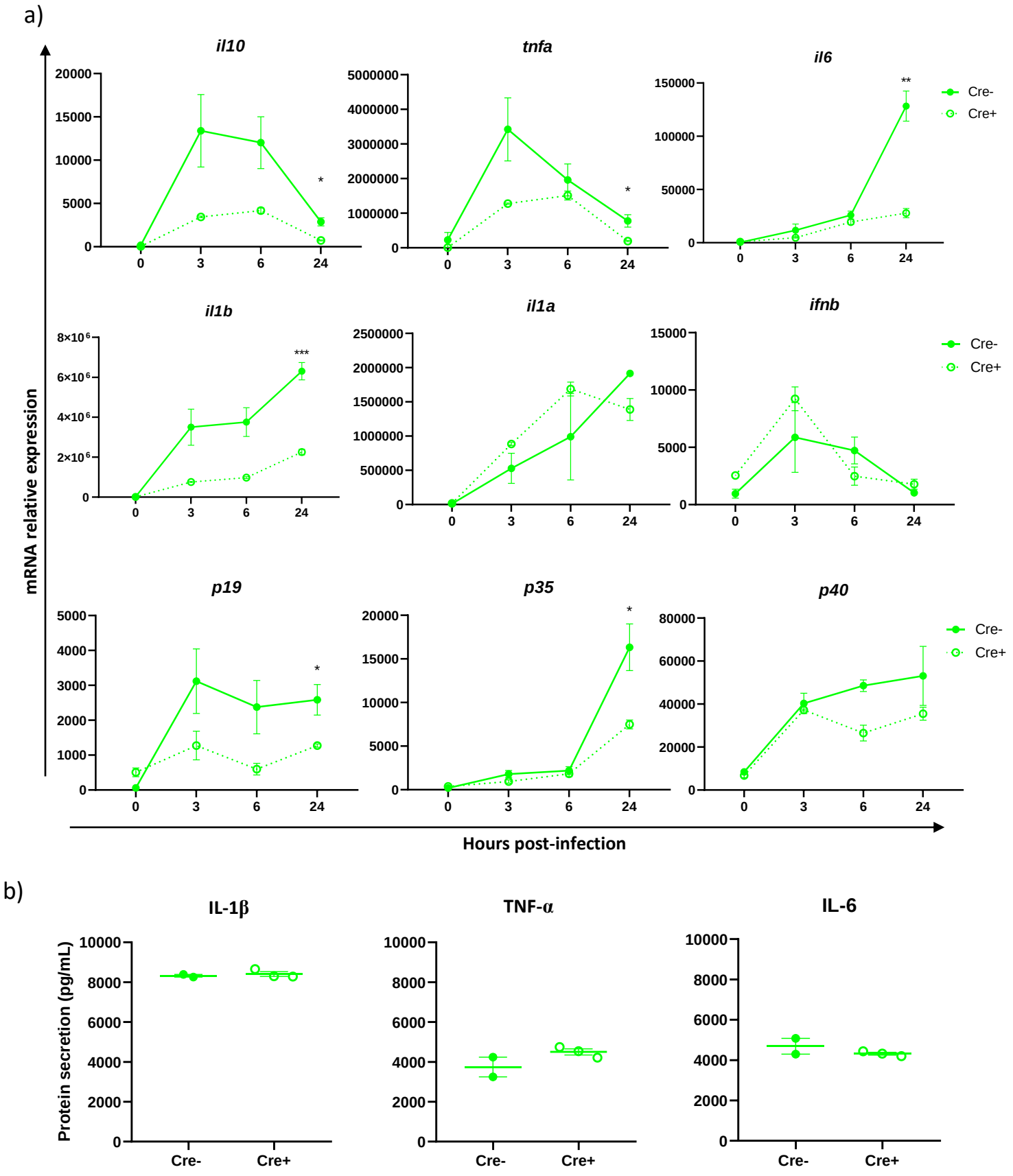

Fig. 24 IL-1R signalling is required within dendritic cells to maintain an appropriate inflammation upon *Mtb* 4I2 infections. IL-1RfloxLyzMCre^{-} and Cre^{+} DCs were generated and infected with isolate 4I2. (a) 3, 6 and 24 hours after infection cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. (b) 24 hours after infection, cell supernatants were collected and the protein levels of the indicated cytokines were measured through ELISA. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05, **p<0.01 and ***p<0.001).

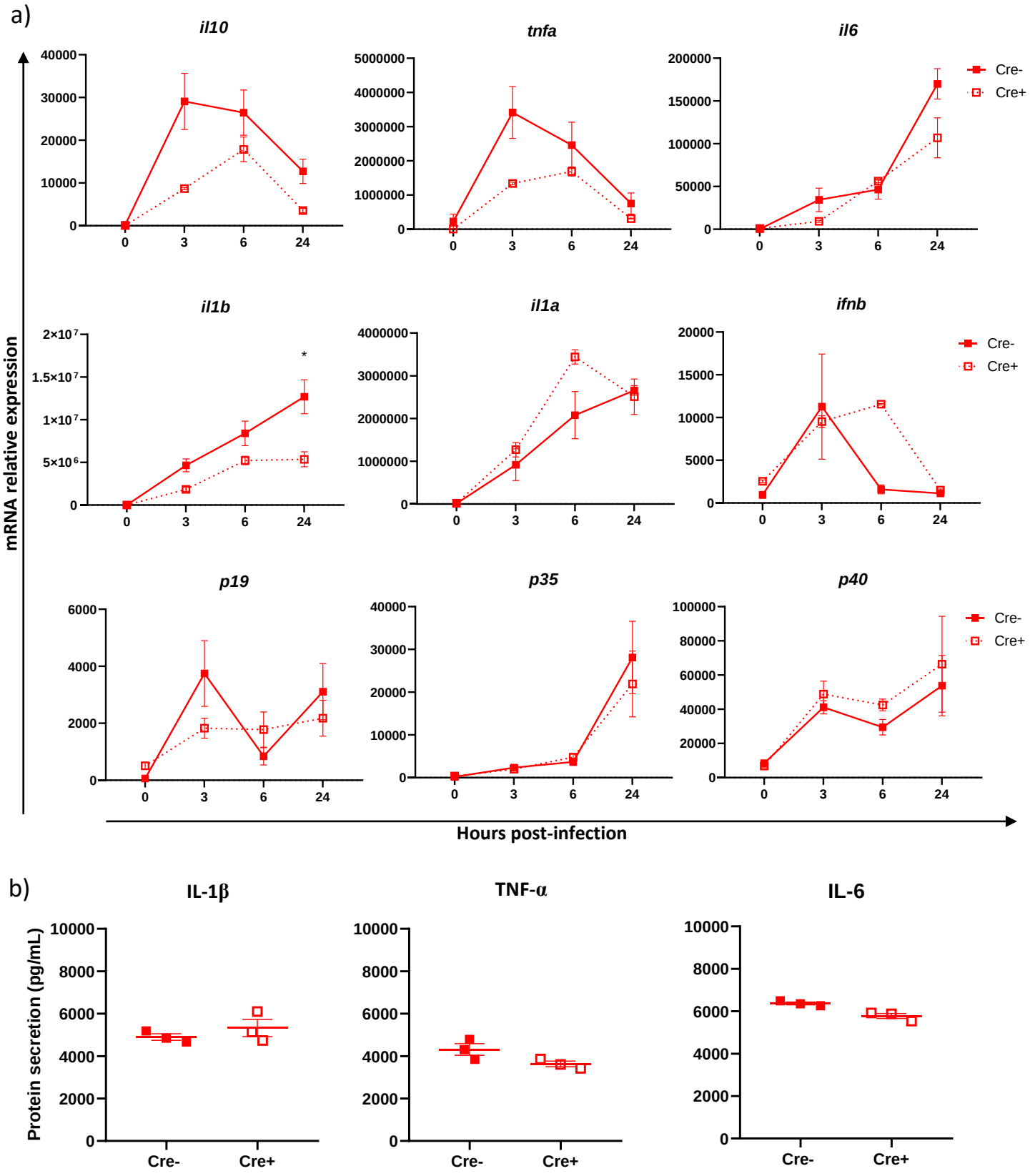

Fig. 25 DCs rely on IL-1R signalling to balance their inflammatory gene expression network upon *Mtb* 6C4 infections. IL-1RfloxLyzMCre^{-} and Cre^{+} DCs were generated and infected with isolate 6C4. (a) 3, 6 and 24 hours after infection cell cultures were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of the indicated panel of cytokines were assessed via qPCR. (b) 24 hours after infection, cell supernatants were collected and the protein levels of the indicated cytokines were measured through ELISA. Represented is mean ± SEM and each symbol represents an individual well. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

Role of IL-23 during infection with *Mtb* 6C4

Considering the exacerbated neutrophil response previously demonstrated within *Mtb* 6C4-infected mice (**Fig. 6**), we questioned whether this phenotype could be related to IL-23 production with subsequent modulation of IL-17-driven responses and granulocyte recruitment. For that, we analysed bacterial burden, gene expression levels, MPO distribution and immune populations from the lungs of *Mtb* 6C4-infected IL-23R$^{-/-}$ mice.

IL-23 is not relevant for the initial control of bacterial growth in the lung

We started by measuring bacterial burden in the lungs of infected animals 30 days post-infection. At this time point, we found the number of lung CFUs to be similar between WT and IL-23R$^{-/-}$ mice (**Fig. 26**). A later time-point of CFUs assessment is currently ongoing, but was not included in this dissertation owing to time restrictions.

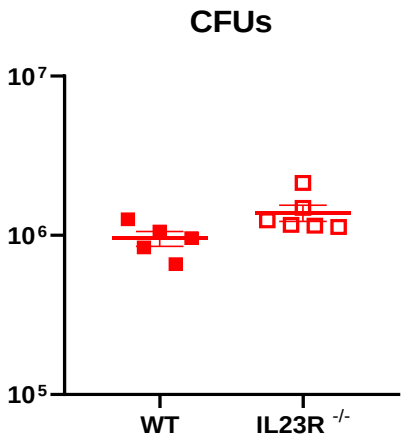

Fig. 26 *Mtb* 6C4 growth control in the lung during the first 30 days of infection does not require IL-23R. C57BL/6 WT or IL-23R$^{-/-}$ mice were infected via aerosol with a high dose (835 CFUs) of *Mtb* 6C4. At day 30 post-infection the lungs were harvested and bacterial load evaluated by CFU enumeration. Represented is mean ± SEM and each point refers to an individual mouse. Statistical analysis was performed using two-tailed unpaired Student's t-test.

IL-23R abrogation does not compromise the transcriptional reprogramming of the infected lung

We next assessed the lung inflammatory microenvironment generated upon infection of WT or IL-23R$^{-/-}$ mice with *Mtb* 6C4, by measuring the expression of genes encoding several inflammatory mediators. The absence of IL-23R did not affect the transcription of most of the tested genes (**Fig. 27a and b**). It was however interesting to notice that IL-23R deficiency negatively impacted the transcription of *il10* and *ifnb* (**Fig. 27c**), suggesting a less anti-inflammatory micro-environment may be generated in these mice. Finally, it is also interesting to highlight that a decreased expression of the IL-23 subunits (p19 and p40) (**Fig. 27d**) was observed in IL-23R$^{-/-}$ mice, suggesting a positive regulatory loop between IL-23R and IL-23 itself.

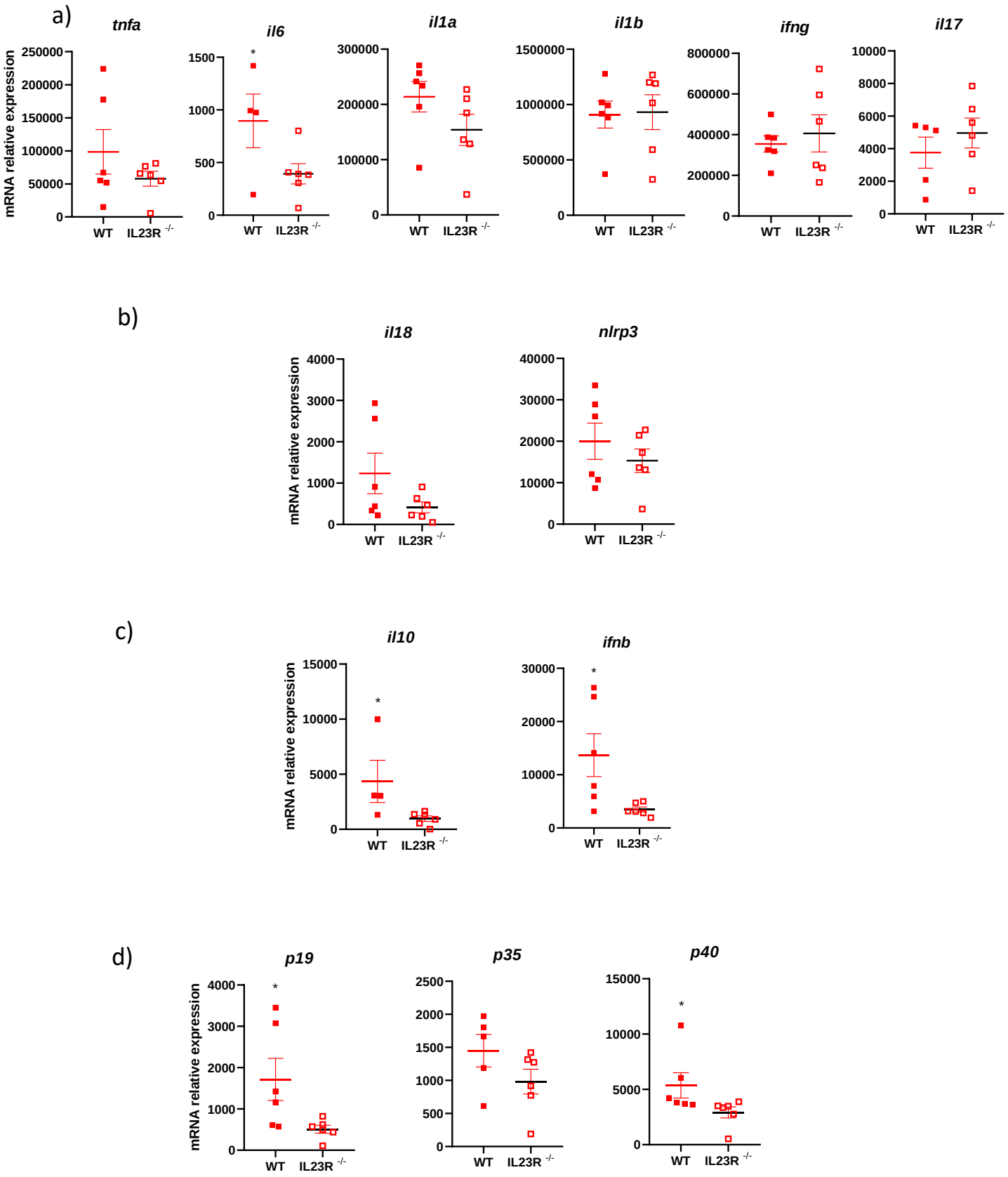

Fig. 27 IL-23R abrogation does not impair genetic responses against *Mtb* 6C4. Harvested lungs at day 30 post-infection with *Mtb* isolate 6C4 from WT or IL-23R$^{-/-}$ mice were homogenized and cells isolated. These were lysed, their RNA extracted and converted to cDNA, after which gene expression levels of inflammatory mediators (a), inflammasome markers (b), anti-inflammatory molecules (c) and IL-12/IL-23 subunits (d) were assessed via qPCR. Represented is mean ± SEM and each point refers to an individual mouse. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05).

IL-23 abrogation increases innate and adaptive immunity and does not ameliorate neutrophil infiltration in the lung

To define the immune cell recruitment to the lungs of WT and IL-23R$^{-/-}$ mice 30 days post-infection with *Mtb* 6C4, we prepared lung cell suspensions and performed a multiparametric flow cytometric analysis of the immune cell populations. The followed gating strategy is presented in **Fig. 28**.

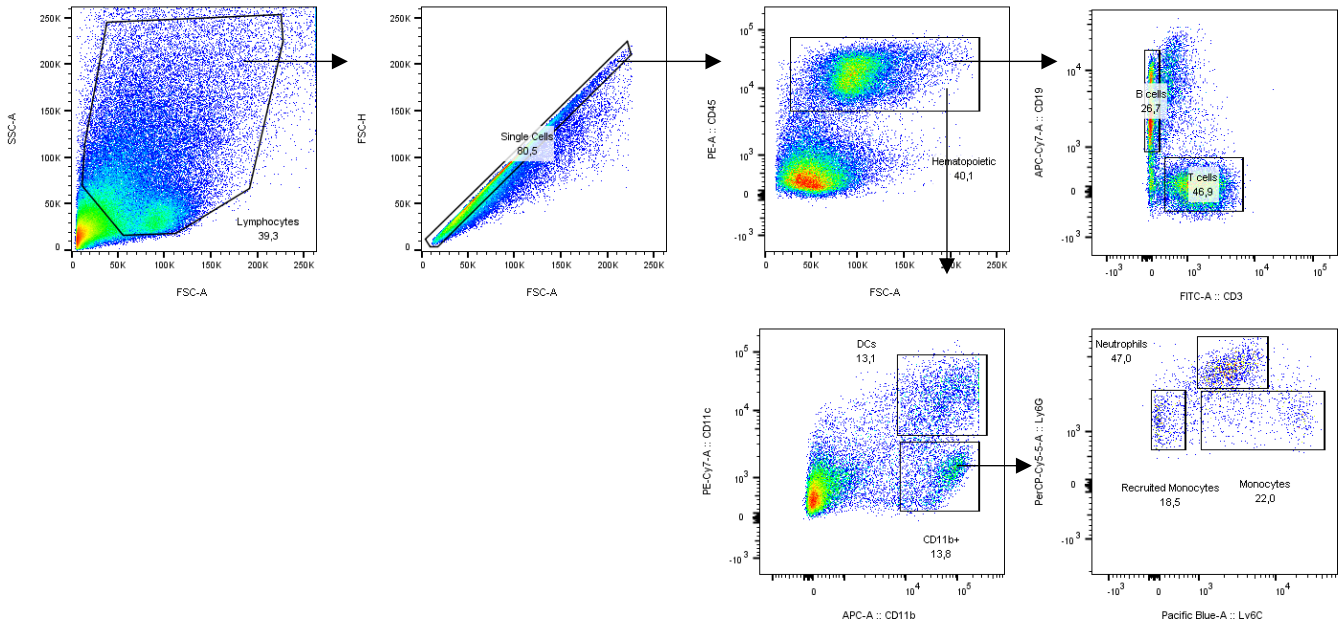

Fig. 28 Gating strategy for immune cell populations within lung suspensions. Immune cell populations from harvested lungs of WT or IL23R$^{-/-}$ mice at day 30 post-infection with *Mtb* isolate 6C4 were determined by flow cytometry. Hematopoietic cells are gated on CD45 expression. B and T cells are gated according to CD19 and CD3 expression, respectively. DCs are positive for CD11c. CD11b^{+} population comprises recruited monocytes, monocytes and neutrophils, which are Ly6G^{-}Ly6C^{-}, Ly6G^{-}Ly6C^{+} and Ly6G^{+}Ly6C^{-}, respectively.

Analysing cell populations, the IL-23R$^{-/-}$ condition revealed an increase in total lung cells (**Fig. 29a**) and immune cell populations (**Fig. 29b and c**). Within the last, statistically significant enhancements of monocytes, recruited monocytes, neutrophils, DCs, B and T cell numbers can be identified (**Fig. 29b**). In line with cell numbers, the frequency of these cell populations was also upregulated in IL-23R$^{-/-}$ mice infected with *Mtb* 6C4, with statistically significant differences for monocytes, neutrophils, B and T cells (**Fig. 29c**). To analyse the extent of neutrophil influx to the lungs of WT and IL-23R$^{-/-}$ mice, MPO immunofluorescences were performed in lung's longitudinal cuts. These revealed a more intense and distributed MPO staining throughout the lungs of IL-23R$^{-/-}$ mice (**Fig. 30**), corroborating the neutrophil augmentation detected by FACS and suggesting higher extents of neutrophil activation following IL-23R inhibition.

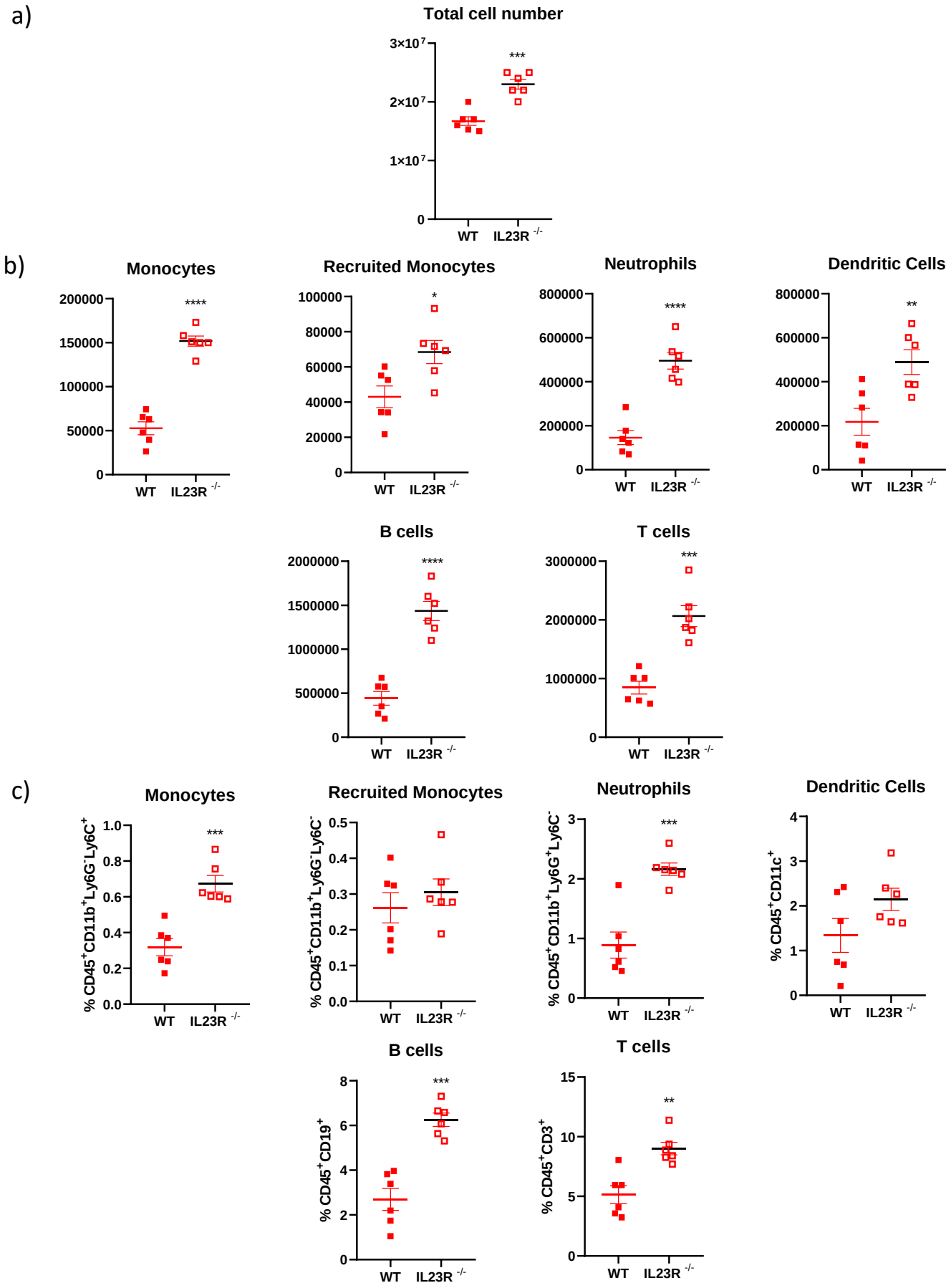

Fig. 29 Inhibition of IL-23R signalling stimulates both innate and adaptive immunity. (a) Following 30 days of infection with *Mtb* 6C4, total cells within lung suspensions of WT or IL-23R$^{-/-}$ mice were counted. Number (b) and percentage (c) of immune cell populations at the same timepoint were determined by flow cytometry. Represented is mean ± SEM and each point refers to an individual mouse. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).

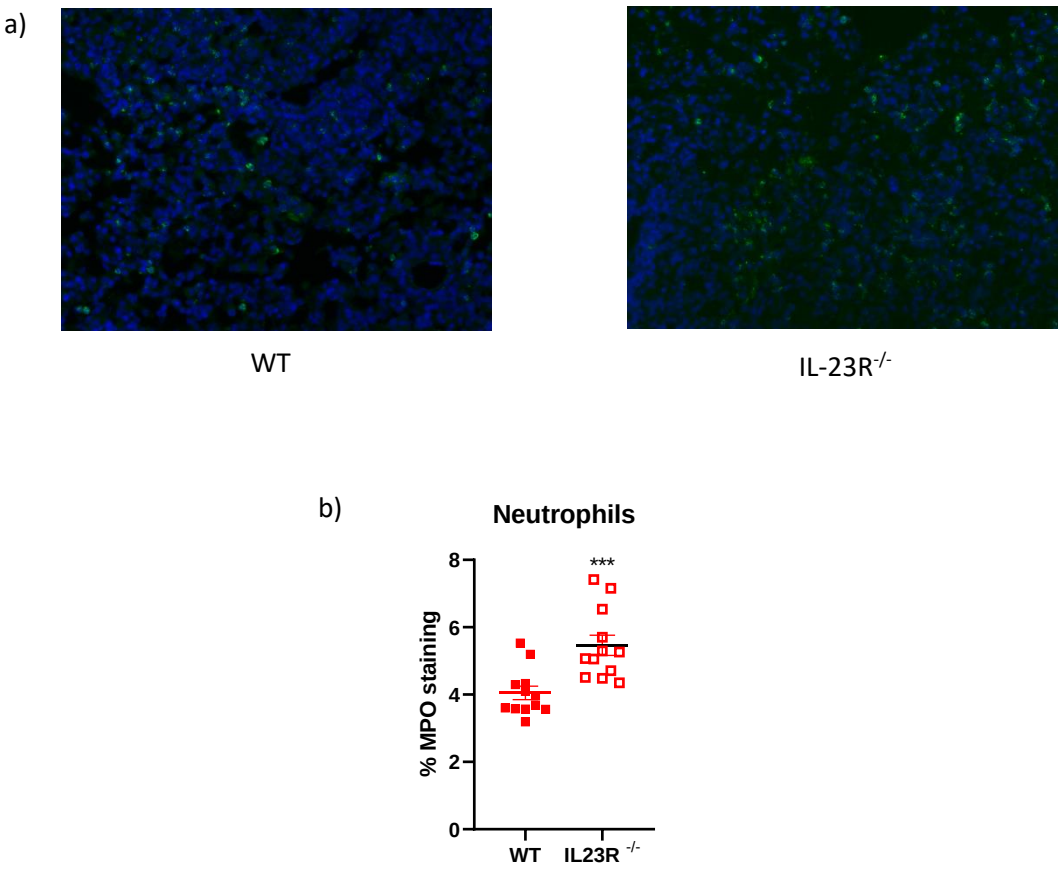

Fig. 30 IL-23R absence increases neutrophil infiltration in the lung. At day 30 post-infection with *Mtb* isolate 6C4 lungs of WT or IL-23R$^{-/-}$ mice were harvested and neutrophil influx determined by MPO immunofluorescence. (a) Representative images of the experimental groups with staining for MPO (green) and DAPI (blue), at 20x magnification. (b) MPO staining was quantified through dividing the number of MPO^{+} cells by the number of DAPI^{+} ones, in 2 image fields per animal. Represented is mean ± SEM and each 2 points refer to an individual mouse. Statistical analysis was performed using two-tailed unpaired Student's t-test (***$p<0.001$).

Chapter 4 – Discussion

Myeloid cells are fundamental for the development of effective immune responses against *Mtb*, for which they require the expression of inflammatory markers, namely IL-1β[100]. Nonetheless, the regulation of its expression levels must be finely tuned, as exacerbated IL-1β drives the progression of pathology and several autoimmune diseases[101,102]. Concerning this, IL-1β modulation appears to be precisely one of the several ways through which *Mtb* isolates causing severe TB evade immune surveillance[93]. Therefore, a detailed understanding of the relationship between IL-1 modulation, IL-1R signalling and the myeloid compartment upon infection with genetically distinct *Mtb* isolates is essential.

AMs comprise the first immune barrier against *Mtb* and, as such, the way these cells interact with the pathogen has a potentially tremendous influence on the outcome of infection. Herein, although AMs demonstrated a similar capacity to control the replication of isolates 4I2 and 6C4, they responded more intensively to *Mtb* 6C4, establishing a genetic increase in their inflammatory network (including IL-1β). This suggests AMs possess negative feedback mechanisms to revert the IL-1β modulation exerted by *Mtb* isolate 6C4. Importantly, we have previously shown that the enhanced IL-1β production by macrophages infected with *Mtb* 4I2 was dependent on the inflammasome activation and not on increased transcription of the *il1b* gene[93]. Since we observed increased transcription of *il1b* in AMs infected with *Mtb* 6C4 but similar protein levels, it is still possible that isolate 4I2 activates the inflammasome in these cells more potently. This is something that requires further investigation. Also, it is possible that other signals might operate *in vivo* that further condition the AM response to these two *Mtb* isolates, as we have evidence that upon aerosol infection with isolate 4I2 increased levels of IL-1β protein are present in the BAL fluid of infected animals (Silvério et al, unpublished). IL-1R abrogation in AMs led to an even more upregulated inflammatory gene expression, although without correlation in terms of protein levels neither bacterial replication control. These findings suggest AMs rely on IL-1R signalling to maintain a balanced inflammatory genetic network but likely not for bacterial containment. Nonetheless, considering isolated cells may not always reflect the pulmonary nature of TB disease, future studies involving more sophisticated systems,

such as microfluidics and co-cultures[103,104], to assess IL-1R signalling in both AMs and alveolar epithelial cells, which are also recognized responders to IL-1[105], are required for a proper understanding of the organism's 1st line of defence against TB.

Monocytes, despite their production of inflammatory cytokines and differentiation capacity into macrophages and DCs[106], have not yet gathered consensus as amplifiers of immune responses during TB[107,108]. Our findings display monocytes as permissive to *Mtb* replication, but also demonstrate that they have an increased genetic expression and protein secretion upon *Mtb* isolate 6C4, which can be analysed as a way for monocytes compensating their low bacterial control capacity. Nonetheless, despite their strong inflammatory transcription, we failed to detect IL-1β protein in cultures of infected monocytes. This result was surprising and requires further validation in future independent experiments. This disconnection between transcription and translation suggests either an inability to activate the inflammasome, as some inflammasome activation pathways are not shared between human and murine monocytes[109,110], or the existence of new host-modulation mechanisms from *Mtb* 6C4 involving the cleavage of pro-IL-1β. In this context, the following observed changes within monocytes' genetic expression might be plausibly viewed as negative feedback mechanisms upon *Mtb* 6C4 infection: i) IFN-β downregulation, considering type I IFN is a negative regulator of IL-1β in *Mtb* infections[69,111]; ii) increase of IL-12 subunits *p35* and *p40* as a commitment of these cells to maintain a strong Th1 response despite a lack of IL-1β, considering monocytes are major IL-12 producers and Th1-response drivers[112,113]. Due to time constraints, the importance of IL-1R signalling with regard to bacterial containment and protein secretion can only be predicted, as no CFU assays nor ELISAs were performed with Cre^+ monocytes.

DCs establish the link between innate and adaptive immunity, owing to their unique capacity of generating distinct T-cell responses. Herein, DCs revealed a versatile role, controlling bacterial replication and strongly inducing genetic and protein inflammatory responses in an IL-1-dependent manner. Within this context, IL-1R signalling abrogation was detrimental to the ability of these cells to control *Mtb* 4I2 and 6C4 replication and launch a strong inflammatory genetic expression, which is consistent with past reports focused on different pathogens outlining that IL-1R-deficient DCs fail to control viral

replication and regulate cell-intrinsic defence genes[114]. Even though future assessments of phagolysosome maturation and reactive oxygen species within *Mtb* 4I2/6C4-infected DCs are of interest, considering the control of bacterial growth from DCs may not always translate into actual killing[115], the present findings encourage research around DCs as targets for improved TB therapies. It will also be interesting to address the impact of the observed differential DC responses in the differentiation of T cells *in vivo*, during infections with these two *Mtb* isolates.

IL-23 boosts IL-17 cellular responses that, when exacerbated, lead to persistent neutrophil influx, unproper bacterial replication and further lung damage[116]. This is a condition seen for instance in the absence of negative regulators of the IL-23 cascade, such as IFN-γ[117], which can be reversed with IL-17 blocking antibodies or p19 subunit inhibition[118]. Considering this, we expected to observe a downregulation of IL-17 expression in the lungs of *Mtb*-infected IL-23R$^{-/-}$ animals, possibly accompanied by decreased neutrophil recruitment. Surprisingly, not only did the genetic expression of IL-17 remain unaffected, but also the distribution of neutrophils throughout the lung increased in the absence of IL-23R. In fact, genetically abrogating IL-23R did not reduce the host's immunocompetence, quite on the contrary: i) the percentage of cells committed to either innate or adaptive immunity increased; ii) the expression of inflammatory mediators (*il1a, il1b, ifng, tnfa, il6 and il17*) and inflammasome-related markers (*il18*, *nlrp3*) was similar between IL-23R competent and deficient mice; iii) control of bacterial replication was not impaired by lack of IL-23R signalling.

Neutrophils usually contribute to lung damage in TB through NET formation[119], which requires MPO production[120]. Despite increased neutrophil recruitment in IL-23R$^{-/-}$, we did not observe increased lung bacterial burdens. We are now analysing the lung histopathology of the infected animals. If no differences are observed in this parameter, it is conceivable that the neutrophils recruited in the IL-23R$^{-/-}$ microenvironment are protective and not detrimental to the host. In this case, it will be important to define their characteristics to envisage molecular pathways that may be modulated to avoid pathological neutrophil responses. Interestingly, considering IL-23 deficiencies appear to dysregulate bacterial control, B cells and IFN-γ production only after 100 days of *Mtb* infection[85], a repetition of our IL-23R$^{-/-}$ experiment with timepoints beyond 30 days and

also involving the following parameters is planned: i) monitoring mice symptoms throughout infection and defining lung scores after mice sacrifice; ii) quantifying other IL-17-producing populations, namely γδ T and natural killer T cells[121]; iii) characterizing $CD4^+$ T cell response beyond CD3 expression to analyse eventual Th1/Th2/Th17 skews. Nonetheless, the herein provided results are noteworthy contributions to the role played by IL-23 in TB and open interesting questions to be pursued in the future.

Chapter 5 – Conclusions

It is now acknowledged that the severity of TB is strain-dependent, as bacteria have evolved strategies to modulate host-pathogen interactions, mainly concerning bacterial replication, IL-1β production and neutrophil recruitment. In this thesis, we used different systems and approaches to better explain the distinct host-*Mtb* interactions, which are evident between 4I2 and 6C4 *Mtb* isolates. Furthermore, our focus was on a thorough understanding of the role of IL-1R signalling in the myeloid compartment and the implications of IL-23R deficiency for the course of infection.

AMs and monocytes are permissive to *Mtb* replication and require IL-1R signalling to maintain balanced inflammatory levels. DCs, out of the assessed myeloid cells, are the most competent in controlling *Mtb* growth and producing inflammatory mediators, abilities which are dependent on IL-1R signalling. The absence of IL-23R signalling within *Mtb* 6C4 infections increases the innate and adaptive immune response, without compromising pro-inflammatory levels neither bacterial growth control.

Despite some of these conclusions requiring further validation, they represent significant contributions to the understanding of *Mtb* 4I2 and 6C4 pathophysiology and prompt future research regarding host-directed therapies for TB.

Chapter 6 – Materials & Methods

Aim 1

Ethics statement

Animal experiments were carried out in strict accordance with recommendations of the European Union Directive 2010/63/EU for the care and handling of laboratory animals, performed in compliance with institutional guidelines and recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) and approved by the National Authority for Animal Health (DGAV).

Animals

Mice aged 8-12 weeks were maintained in specific pathogen-free conditions in the i3S animal facility with controlled temperature (20-24°C), humidity ranging between 45-65%, 12-hour light/dark cycles and *ad libitum* food and water. Animals were euthanized by intraperitoneal injection of a lethal dose of pentobarbital (200 µl).

Alveolar macrophages

Mice's tracheae were exposed and a small incision was performed. A 1 ml syringe was filled with 37°C pre-warmed BAL buffer (PBS + 0.5% FBS + 0.4% EDTA) and a needle-free catheter was inserted on its tip. The catheter was then introduced inside the trachea, the buffer pushed and retrieved three times into the alveolar space and the retrieved volume collected into a 5 ml tube. This process was repeated three times. Following centrifugation, the supernatant was discarded and erythrocytes were lysed with 1 ml of ACK lysis buffer and subsequent BAL was filtered through 70 µm strainers. Cells were then centrifuged, resuspended in cRPMI (RPMI + 1% Glutamate + 1% HEPES + 10% FBS) and further plated in 96-well plates at a density of 1*10^5 cells/well. AMs were then used to assess CFUs (4 hours and 4 days after infection), mRNA expression (6 hours after infection) and cytokine production (24 hours after infection).

Monocytes

Cells were isolated from bone marrow of the tibia and femur. The tips of these two bones were cut and bone marrow was collected by centrifugation. Erythrocytes were lysed with ACK lysis buffer and cells were filtered through 70 µm strainers and resuspended in cDMEM (DMEM + 1% Glutamate + 1% HEPES + 10% FBS). Monocytes were then magnetically isolated using EasySep Mouse Monocyte Isolation Kit (Stemcell Technologies) according to the manufacturer's instructions and further plated in 24-well plates at a density of 5*10^5 cells/well. Monocytes were used to assess CFUs (4 hours and 4 days after infection), mRNA expression (6 hours after infection) and cytokine production (24 hours after infection).

Dendritic cells

DCs were derived from centrifuged bone marrow as previously described. Bone marrow cells were plated in T75 flasks at a density of 2*10^5 cells/mL using 25mL of cRPMI supplemented with 20 ng/mL of recombinant mouse GM-CSF (BioLegend, Cat# 576306). Three days after plating, 25mL of fresh cRPMI supplemented with 20 ng/mL of GM-CSF were added to the culture. On days 6 and 8, half of the cell suspension was recovered from the flasks, resuspended in fresh cRPMI supplemented with 20 ng/mL of GM-CSF and introduced back to the original flasks. Cells were harvested on day 9 and plated in 24-well plates at a density of 5*10^5 cells/well. DCs were then used to assess CFUs (4 hours and 4 days after infection), mRNA expression (6 hours after infection) and cytokine production (24 hours after infection).

In vitro infections

Mycobacterial clumps were dissolved by gentle passaging through a 25G needle, mixed within respective cell medium and added to the wells of *in vitro* cultures at a MOI of 2.

Experimental infections

Mice were infected with a high dose of nebulized bacteria via aerosol route, using a Glas-Col inhalation system. Infection dose (835 CFUs) was attained by determination of the number of viable bacteria in mice's lungs 3 days after infection.

Lung processing

Thirty days after aerosol infection, lungs were perfused with 10 mL of PBS through the heart's right ventricle. Following excision, the right upper lung lobe was fixed in 2% paraformaldehyde (PFA) for immunofluorescences. The remaining lobes were mechanically disrupted, followed by digestion in collagenase at 37 °C for 30 minutes. Lungs were then homogenized through 70 µm strainers into cell suspensions that were used for CFU quantification, mRNA expression assessment and FACS.

CFUs assay

In vitro cultures and lung cell suspensions were lysed with 1% saponin to release intracellular bacteria. Ten-fold serial dilutions of saponin-treated cell suspensions were then further plated in Middlebrook 7H11 agar plates. Following incubation for 21 days at 37°C, colonies were counted under a magnifier lens.

RNA isolation

In vitro cultures and lung cell suspensions were lysed with TripleXtractor (GRisSP Research Solutions) and RNA extraction performed according to the manufacturer's protocol.

cDNA synthesis

cDNA was synthesized from RNA using the ProtoScript First Strand cDNA synthesis kit (New England Biolabs) and following manufacturer's instructions.

qPCR

Targeted genes *il10, tnfa, il6, ifnb, il1b, il1a, nos2, il17, ifng, il18, nlrp3, p19, p35* and *p40* were quantified by qPCR with SYBR green (Invitrogen) and normalized to ubiquitin mRNA levels. Gene expression analysis was performed using Bio-Rad CFX Maestro 2.2.

Cytokine detection

Cytokines were detected in supernatants of infected cultures by ELISA for IL-1β (Invitrogen, 88-7013), TNF-α (Invitrogen, 88-7324) and IL-6 (Invitrogen, 88-7064) according to the manufacturer's instructions.

Fluorescent-activated Cell Sorting

DCs and lung cell suspensions were previously cleared of erythrocytes with ACK lysis buffer and resuspended in FACS buffer (PBS + 2% FBS). DCs were stained for surface antigens with the following antibodies: CD11c-PE-Cy7 (Clone HL3, BioLegend), CD80-APC (Clone 16-10A1, BioLegend), CD86-PE (Clone GL1, BD Biosciences) and MHCII-PerCP-Cy5.5 (Clone M5/114.15.2, BioLegend). Lung cell suspensions were stained for surface antigens with the following antibodies: CD3-FITC (Clone 17A2, BioLegend), CD11b-APC (M1/70, BD Biosciences), CD11c-PE-Cy7 (Clone HL3, BioLegend), CD19-APC-Cy7 (Clone 6D5, BioLegend), CD45-PE (Clone 30-F11, BioLegend), Ly6C-Pacific Blue (Clone HK1.4, BioLegend) and Ly6G-PerCP-Cy5.5 (Clone 1A8, BioLegend). Following fixation with 2% PFA, cells were further resuspended in FACS buffer and acquired on a BD FACS Canto II. Data analysis was performed in FlowJo v10.8.

Immunofluorescence

Following fixation in 2% PFA, the right upper lung lobe was sectioned in 3 um-thick slices and stained for MPO (purified goat IgG, #AF 3667, R&D Systems) overnight. Secondary antibody (anti-goat IgG, Alexa Fluor 488, Invitrogen) was incubated for 1h and DAPI was used for counterstaining. Square coverslips were mounted in Vectashield mounting medium and examined under a confocal microscope (Zeiss Axio Imager Z1). Digital images were acquired with Zeiss software. Cell quantifications were performed with automated counting of single color images using ImageJ.

Mtb media preparation

7H11 agar medium was prepared with 7H11 Middlebrook powder, 0.5% glycerol and 10% oleic-acid-albumin-dextrose-catalase supplement, according to the manufacturer's protocol. BBL MGIT PANTA antibiotic mixture (BD Bioscience) was added to the solution to prevent contaminations.

Statistical Analysis

Data were analysed with GraphPad Prism 8.0.1 software. Differences between groups were assessed with unpaired, two-tailed Student's t-test. Differences were considered significant for $p<0.05$ and represented as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

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