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Cytokine-mediated accrual of FoxP3^{-} IL-10^{+} CD4 T-cells with age and their impact on immune response to infection with experimental *Mycobacterium tuberculosis*

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ABBREVIATIONS

Abs	Antibodies
AhR	Aryl-hydrocarbon Receptor
APCs	Antigen Presenting Cells
BAL	Bronchoalveolar lavage
BCL	B-Cell lymphoma
Blimp-1	B lymphocyte-induced maturation protein-1
BMDC	Bone marrow derived dendritic cells
CRP	C-reactive protein
COVID-19	Corona virus disease-19
DCs	Dendritic Cells
DCAF	Dbb1 and CUL4 associated factor 1
DUSP	Dual Specific Phosphatase
EGR2	Early Growth Response 2
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular-signal Regulated Kinases
FoxP3	Forkhead box P3
GC	Germinal centre
sgp130	soluble Glycoprotein 130
HSC	Haematopoietic Stem Cells
HyIL-6	Hyper-IL-6
ICOS	Inducible T-cell COStimulator
IFNγ	Interferon gamma
IGRA	Interferon-gamma release assay
IL	Interleukin
sIL-6Rα	soluble Interleukin-6 receptor alpha
ILT	Immunoglobulin-like transcript
IVC	Individually ventilated cages
JAK	Janus tyrosine kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MHC	Major histocompatibility class

MMP	Matrix Metallo Proteinase
Mtb	*Mycobacterium tuberculosis*
mths	Months
MuFTs	Multifunctional T-cells
NK	Natural Killer
PI3K	Phosphoinositide 3-kinase
RANKL	Receptor Activator of Nuclear factor Kappa-B ligand
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RCB	Research Centre Borstel
RORγt	Retinoic acid receptor-related orphan nuclear receptor gamma t
ROS	Reactive Oxygen Species
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
SAA	Serum Amyloid A
SASP	Senescence-associated secretory phenotype
SIRT1	Sirtuin-1
SNPs	Single Nuclear Polymorphisms
SOCS	Suppressor of Cytokine Signalling
SSC	Senescent somatic cells
STAT	Signal Transducer and Activator of Transcription factor
TB	Tuberculosis
TCR	T-cell Receptor
Teff	Effector T-cells
TFH	T-follicular helper
TGF-β	Transforming growth factor beta
TH	T-Helper
TNF	Tumour necrosis factor
TR1	Type 1 Regulatory T-cells
exTH17	ex-T-helper 17
exTregs	ex-Regulatory T-cells
WHO	World Health Organisation
Wks	Weeks
WT (C57BL/6)	Wild type

1 INTRODUCTION

1.1 Aging and Immunosenescence

The advent of modern medicine, improved hygiene and nutrition have led to a steady rise in the number of individuals aged 65 and above, thus changing the demographic of our society. A 2010 study by the world health organisation (WHO) predicts that the number of individuals aged 65 and above will grow from 524 million to approximately 1.5 billion by the year 2050, with most of this increase occurring in industrialised nations[1]. Now, most individuals who boast of longevity do not necessarily enjoy the benefits of a healthy lifespan, so it is safe to say that health systems will be burdened with diseases of old age in the near future. The question that now arises is whether we will have the capacity to accommodate this aging population.

The effect of aging on morbidity or healthy life span is heterogenous, as no 2 individuals age in the same way. Lopez-Otin et al., characterised cellular and molecular aging using the following 9 hallmarks: genomic instability, shortening telomere length, epigenetic modifications, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intracellular communication[2], all of which impact the immune system. Therefore, aging can be described as a process in which the build-up of deficits results in immune dysregulation and reduced vitality in individuals. This immune dysregulation stems from changes that occur in both the innate and adaptive immune system, culminating in increased autoimmune disorders due to impaired immune tolerance, increased chronic inflammation as a result of impeded clearance of senescent cells, ineffective vaccine response, and increased susceptibility to diseases and infections in the elderly[3], as evidenced by the ongoing coronavirus disease (COVID)-19 pandemic, where death rates are as low as 0.1% in children but increases to about 10% in older individuals[4,5]. This increased susceptibility as well as mortality to infections in the elderly[6,7] is an attest to the decline in fidelity and efficiency of aged immune systems, known as “immunosenescence”.

The cause of immunosenescence is multifactorial and includes factors such as thymic involution, clonal expansion of antigen-experienced cells in favour of naïve cells and stem cell exhaustion[8,9], which ultimately favour the production of proinflammatory mediators[10,11].

Given that dysregulation of immune function is central to most of the biological changes associated with aging and age-related diseases, this introductory section will summarise

current knowledge on how the elderly respond to infections, particularly of the lung, and how the changes that occur in the immune system with age underpin the weakened immune response in older individuals.

1.2 Consequence of aging on infectious diseases

Infections remains the primary cause of death for a third of individuals aged 65 and above, because altered immune status and various physiological changes (Tab. 1) increases their predisposition to infectious diseases[12,13]. In the lungs, for example, increased alveolar size and alveolar capillary area, and the deterioration of excretory mechanisms such as cough or mucociliary clearance[13] further increases the risk of lower respiratory tract infection. In addition, clinical presentation of disease is altered in elderly individuals, further complicating diagnosis and treatment.

Table 1. Changes in Lung anatomy and immune responses that increase susceptibility to infections

Factor increasing predisposition	
Lung elasticity↓[12]	Lymphocyte numbers↓[13]
Mucociliary clearance↓[13]	Vaccine response↓[13]
Glottic closure response↓[12]	Inflammatory cytokines↑[13]
Cough/sneezing↓[12]	
Fever↓[13]	

Infections, particularly of the lower respiratory tract have a predilection for older individuals, with higher morbidity and mortality rates being the norm compared to younger individuals. A study in Norway using a notification rate ratio (NRR) showed that pneumococcal infections are more prevalent in the elderly population: About 50% of community-acquired pneumonia cases occur in individuals older than 65 years of age, and a third of them develop severe sepsis[14]. Age-related changes in immune surveillance, particularly reduced innate and adaptive immune responses may be responsible for increased lung pathology in response to infective agent. Additionally, chronic inflammation in the aged lung increases susceptibility by upregulating receptors such as keratin 10 (K10), laminin receptor (LR), and platelet activating factor receptor (PAFr) on the surface of epithelial cell, which increases adhesion and accumulation of bacteria in the lung[15].

Following closely behind are viral infections of the respiratory tract, which are also responsible for a large percentage of deaths in the elderly. This is exemplified in seasonal influenza cases, where most severe cases with hospitalisation and deaths are in the age group ≥ 65. Viral infections also render the elderly susceptible to re-infections with pneumococcal bacteria[16].

The effects of immune aging on lower respiratory tract infections are yet to be fully elucidated. What we do know is that there is a difference in immune fitness between young and the elderly, thus establishing advanced age as a risk factor for lung infections

1.2.1 Tuberculosis in aging

Tuberculosis (TB), one of the leading infectious causes of death worldwide, caused by *Mycobacterium tuberculosis* (*Mtb*), continues to claim more than 4000 lives every day, according to the WHO. Although most individuals are latently infected with *Mtb* (asymptomatic), reactivation can occur when the immune defence is weakened. A major risk factor for contracting TB is increasing age, as elderly individuals are more likely to suffer a primary infection with adverse effects[17].

Mtb resides within the lung mucosa (LM) of the alveolus during infections. For proper lung maintenance and function, the LM is recycled frequently by the alveolar epithelial. In old age however, LM recycling is impeded, resulting in an accumulation of old LM cells and an increase in inflammatory mediators in old lungs[18], which alters the functions of many innate defence mechanisms. For example, dipalmitoylphosphatidylcholine (DPPC) which binds to and activates surfactant protein A (SP-A) is reduced in the lungs of elderly individuals, thereby decreasing receptor expression on macrophages and rendering phagocytosis of *Mtb* ineffective[18,19]. Furthermore, one study identified autophagy as a means to suppress *Mtb* survival within intracellular compartments[20], but reduced autophagy in advanced age negates this defence mechanism.

Cellular immune response to *Mtb* infection involves not only cells of the innate immune system, but also CD4 and CD8 T-cell responses that kill or contain the bacteria in granulomas. However, reduced CD4 T-cell output and thus the impaired generation of antigen-specific cells[21-23], the shift from T-helper (TH) 1 to a TH2 type immune response in milieus with high interleukin (IL)-6[24] and ineffective phagocytosis by macrophages[25] adversely affect the *Mtb*-specific immune response in old age. Consistent with this, higher bacterial burdens were measured in spleen cell cultures from aged mice[23]. Moreover, when the T-cells from these old mice were transferred into young mice, they failed to protect young mice from a challenge with *Mtb*[23]. Additionally, there is evidence to suggest that the persistent low-grade inflammatory state in elderly individuals may also contribute to the immunopathogenesis of TB by providing a supporting environment for bacterial replication. Indeed, when nitric oxide repressed proinflammatory IL-1-dependent 12/15-lipoxygenase neutrophil recruitment

pathway in mice, bacterial burden was reduced[26]. These results from *in vitro* and *in vivo* mouse studies mirror reports on the outcome of *Mtb* infections in the elderly[17].

Senescence associated immune decline remains a risk factor for adverse outcomes of diseases and infections in the elderly. For the longest time, research focused on the changes that occur at cellular levels. More recently, the influence of cytokines on immune aging has been recognised, but the exact cells that produce the cytokines (e.g. after *Mtb* infection) that alter cell differentiation and contribute to the weakened immune response in advanced age remain elusive.

1.3 Immunological changes during aging

A striking change associated with aging is the decline in immune function, with the immune system becoming less able to identify foreign antigens. While this by no means implies that older people are immunocompromised, the immunological changes that accompany aging describe a compromise in homeostatic equilibrium, resulting in alterations in pro- and anti-inflammatory mediators that eventually dampen immune responses.

1.3.1 Changes to the innate immune cells

As a first line of defence, the innate immune system is important in defending against invading pathogens and does not require antigen specificity. Its main cellular components being macrophages, neutrophils, dendritic cells (DCs), and natural killer (NK) cells are affected by aging. Changes to cells of the innate immune system with age are not as extensively studied as those of the adaptive immune system, but a typical trait reported on is a switch from a quiescent to a perpetually activated innate immune system[27]. This age-associated activation is thought to be induced by factors such as the spontaneous production of reactive oxygen species (ROS), dysregulation of autophagy, and the release of endogenous damage-associated molecular patterns (DAMPS)[28,29]. For example, Hazeldine et al., showed a link between altered ROS production and the inability of neutrophils to produce extracellular DNA traps (NETosis)[30] thus, impairing neutrophil anti-bacterial function. Furthermore, neutrophils in the elderly are prone to death as their sensitivity to *N*-formyl-methionyl-leucyl-phenylalanine (*N*-fMLP), which extends their lifetime at infection sites, declines with age[31,32]. Because of impaired autophagy, these neutrophils accumulate and increase inflammation thereby causing tissue damage and favour tumorigenesis.

When natural killer (NK) cells were examined, production of cytokines from these cells were altered with age, as studies in humans have shown a decline in $CD56^{bright}$ (cytotoxic) and an increase in $CD56^{dim}$ (cytokine producers) NK cells subsets[33,34] which would account for the skewed or dysregulated cytokine production with age. Not only is cytokine production from NK cells skewed but reports of reduced chemokine production such as C-C motif ligand 3 (CCL3), IL-8, regulated on activation normal T cell expressed and secreted (RANTES) in individuals aged 90 and above have been published[35]. This decreased chemokine production might explain the shift towards a TH2 responses observed in elderly. In vaccine studies, altered NK cell activity from older test subjects was associated with poor seroconversion[33], suggesting that impaired NK cell function may have broader effects that increase susceptibility to viral infections and cancers in the elderly.

Research on the effects of aging on macrophages has not yielded clear cut answers. Some studies report an upregulation while others report on a downregulation of these immune cells. Human studies with macrophages demonstrate an increase in proinflammatory cytokines including IL-6, tumour necrosis factor (TNF) and IL-1 in peripheral blood with advancing age[36]. *In vitro* mouse studies with lipopolysaccharide (LPS) stimulation shows a decrease in proinflammatory cytokine and a subsequent increase in anti-inflammatory cytokine production[37]. The study also implicates the reduction in the number of cells expressing CD14 and Toll-like receptor 4 (TLR4) as the reason for reduced proinflammatory cytokine production[37]. This reduction in proinflammatory cytokines hinders the effective activation of B-cells by macrophages. Conversely, the production of proinflammatory cytokines after *in vivo* LPS stimulation increases in aged mice[38]. This discrepancy between both studies might be due to the action of other stimuli present in the host's microenvironment such as hormones, chemokines, adipose tissue etc. that change with age, therefore, the function of macrophages *in vivo* can be considerably altered in response compared to their *in vitro* response. Aging is also associated with an accumulation of the proinflammatory M1-macrophage subset that have impaired phagocytosis and cytokine production[25].

Studies in aged DCs also provide conflicting results, most likely as a result of the differences in experimental setups and DC populations tested. While some studies record a stability in conventional DC (cDCs) numbers in blood overtime[39,40], others report a decline in cDCs numbers and decreased secretion of proinflammatory cytokines in the elderly when compared to younger subjects[41]. Agrawal et al., demonstrated impaired phagocytosis of

foreign organisms by monocyte derived DCs (moDCs) from aged individuals. They also identified reduced activation of the Akt kinase in the phosphoinositide 3-kinase (PI3K) signalling pathway as the culprit for reduced DC phagocytosis[40]. Another observation made was the increased reactivity to self-antigen by DCs from older individuals, resulting in autoimmunity and chronic inflammation. In terms of antigen stimulation of T-cells, one study found that cytokine secretion from moDCs of young and old subjects were comparable[42], whereas another study found that moDCs from older subjects stimulated with LPS produced more proinflammatory cytokines[40]

Alterations in cytokine secretion and ineffective uptake of pathogens hinders the successful activation of the adaptive immune response thereby increases the occurrence of infectious illnesses in aged individuals.

1.3.2 Changes to the adaptive immune cells

The adaptive immune system is equipped with an immensely diverse repertoire of antigen-responsive T- and B-cells that upon antigen stimulation, proliferate to produce antigen-specific lymphocytes, which then become less proliferative effector and memory cells. On antigen reappearance, these memory cells secrete antibodies, cytokines etc. to tackle foreign antigens and protect the host. As one ages, naïve T- and B-cell output diminishes thereby reducing adaptive cells repertoire and creating a selective pressure towards proliferating antigen-experienced cells. Not only are the immune cells affected by aging, reports show changes in the stroma of both the thymus and bone marrow[43,44] resulting in suboptimal responses of the cells to growth and development factors within these compartments. All these make the immune system highly susceptible to aging processes.

1.3.2.1 B-Lymphocytes

Antibodies produced by B-cells play a crucial role in controlling or clearing invading pathogens. Like the other immune cells, B-cell numbers and functions are also affected by aging. Stephan et al., showed that pre-B-cells in the bone marrow were reduced by up to 90% in old mice and this reduction occurred during the transitioning stage from pro-to pre-B-cells[45]. Published data also highlights the role of inflammation in altered B-cell development with age. One found an accumulation of senescent cells in the bones of old mice and humans due to faulty autophagy clearing mechanism[46]. These amassed senescent cells subsequently increase infiltrating pro-inflammatory mediators[47,48]. Inflammation also biases haematopoietic stem cells (HSC)

differentiation towards myeloid cells at the detriment of lymphoid cell differentiation, thus resulting in the decline in B-cell output from the bone marrow with age[49,50]. Furthermore, bone marrow cellularity is altered in aging, resulting in the conversion of HSCs to fatty marrow cells. Indeed, IL-1α and IL-1β from adipocytes within the bone marrow increased myeloid cell differentiation[51]. Changes in the bone marrow microenvironment lead to a decline in IL-7, an important cytokine for expansion and survival of B-cell precursors in mice[44], whilst cytokines such as IL-21, IL-4 and interferon γ (IFNγ) trigger the accumulation of age-associated B-cells at the expense of follicular B-cells in spleen and visceral adipose tissue of old mice[52,53].

However, discrepancies in reported results in human studies, due to differences in experimental set up, have made it difficult to draw a conclusion on how B-cell populations are affected in aging[54,55]. One can assume that B lymphopoiesis reduces with age, since portions of haematopoietic bone marrow is replaced by fat cells. Another thing we can draw from mouse studies is the accumulation of age-associated B-cells. These cells are also identified as the IgD^- $CD27^-$ memory B-cell population and are often found in elderly individuals with autoimmune diseases such as rheumatoid arthritis[56]. They are good antigen presenters but favour a polarisation of TH17-cells[57], which could further exacerbate damage to B-cell compartments by increasing overall inflammation.

In addition to the reduced B-cell numbers, there is also a decline in the quality (affinity) of antibodies produced, which can be traced back to altered B-cell metabolism with age[58]. It was seen in the study by Kurpati et al., aged B-cells had defective mitochondria, reduced energy output, and increased mitochondrial ROS (mROS) production[58]. Increased mROS leads to increased glycolysis and the subsequent reduction of sirtuin (SIRT)-1 expression[59] which is important for antibody specificity. This was made clear when SIRT1 expression, regardless of age, was investigated and individuals with reduced expression had significantly reduced trivalent inactivated influenza vaccine specific antibody responses, compared to those with high SIRT1 levels[58]. In addition to low quality antibodies, B-cells from the elderly are unable to undergo effective class-switching as a result of low expression of cytidine deaminase[60,61]. Transcriptomic results of *in vitro* B-cell cultures showed marked reductions in transcripts for one-carbon metabolism in activated aged B cells[58]. One-carbon metabolism is important for synthesis of amino acid, nucleotides, DNA methylation and redox defences[62]. This implies that B-cell generation and its epigenetic regulation are diminished in elderly individuals resulting in an overall decrease in B-cell numbers with age.

1.3.2.2 T-Lymphocytes

Able to detect foreign- and neo-antigens, T cell are key players in orchestrating adaptive immune responses, such as humoral immunity development, memory cell generation etc. Their development also begins with haematopoietic stem cells in the bone marrow that then develop into lymphoid progenitor cells. Progeny destined to become T-cells emigrate out of the bone marrow to the thymus where all further developmental steps occur.

As one ages, the epithelial tissue in the thymus is slowly replaced by adipose tissue, a phenomenon seen in the vast majority of vertebrates that describes the thymic involution process[21,22]. As a result of thymic involution, naïve T-cell output declines and decreases T-cell receptor (TCR) repertoire. Surprisingly, although naïve T-cells are diminished the overall T-cell numbers in the periphery remains unchanged throughout different age groups[63]. The homeostasis in T-cell numbers might be due to compensatory mechanisms that correct for the reduced thymic naïve cell output. For example, circulating naïve T-cells from older individuals were reported to express less of the pro-apoptotic molecule BIM[63], thereby increasing their survival.

With age, there is an inverted ratio of CD4 to CD8 T-cells deficient of the co-stimulatory marker CD28[64]. These CD4 T-cells cannot be properly primed, therefore do not generate intact CD4 TH cell subsets, instead they produce large amounts of pro-inflammatory cytokines. Likewise, an increase in clonally expanded memory T-cell pool[64] due to repeated viral antigen exposure is a characteristic of aged T-cells. Indeed, cytomegalovirus (CMV) infection is associated with the expansion of terminally differentiated CD8 (T_{EMRA}) effector memory cells[65]. In addition to homeostatic dysregulations, alterations in T-cell functionality occurs with age. There is reduction in proliferative capacity and an ineffective signalling of T-cells[66]. For example, *in vitro* studies recognised impaired extracellular signal-regulated kinase (ERK) phosphorylation and reduced TCR sensitivity to antigens, due to a decline in miR-181a expression. miR-181a controls the activation of ERK negative feedback pathways like dual specific phosphatase (DUSP) 5 and 6 or SIRT1 pathways[66]. Collectively, the aforementioned changes contribute to the defects seen in T-cells in the elderly.

1.3.2.2.1 TH1/TH2-cells

The TH cells are not spared from the changes associated with the immune aging. TH1- and TH2-cells were first identified in mice by Mosmann and Coffman[67] and then in humans by Romagnani[68]. TH1-cells secrete IL-2 and IFN-γ, tend to provide help for CD8-mediated

cytotoxic effector function and IgG2a antibody to defend against intra cellular pathogens, whereas TH2-cells secrete IL-4 and IL-10 and are more efficient in providing help during humoral immune response against extracellular parasites, allergies or autoimmune diseases[69]. Even in healthy aging, CD4 T-cells are committed to a TH2 phenotype[70]. This shift towards a TH2 immune response influences the effectiveness of control of cancers or infections like tuberculosis[24], as both ailments require TH1-mediated immune responses for their control. In tumours, CD4 T-cells supports eradication by activating other immune cells and increasing IFNγ production from TH1-cells in the tumour's microenvironment. However, in aged patients, TH2-cells rather than TH1-cells are predominantly detected[24,70], thus, creating a permissive environment for cancer development. Many factors are involved in the shift towards a TH2 phenotype with age. One study points to the effect of the IL-6/IL-21 axis, whose activity increases with age, resulting in dampened TH1 response[24,71]. The differentiation of CD4 T-cells to TH1-cells are also affected by a downregulated glutathione redox system, because when glutathione is downregulated, it is unable to stimulate IL-12 production, which is important for the differentiation of TH1-cells[72]. Since CD4 T-cells predominantly differentiate to TH2-cells in old age, one could assume that they are the source of increased IL-10 production but this still needs to be investigated.

1.3.2.2.2 TH17-cells

TH17-cell subset of CD4 T-cells, produce IL-17 family cytokines (IL-17A—F). Of this family, one of the most studied is IL-17A that plays an important role in defending against fungal infections and exacerbating chronic inflammatory and autoimmune disorders[73]. IL-17A recruits neutrophils to sites of infection by induction of IL-8 and granulocyte-colony stimulating factor (G-CSF) production from epithelial cells and fibroblasts. Indeed, IL-17 receptor A deficient mice had compromised neutrophil recruitment and increased susceptibility to fungal infection[74]. IL-17 requires for its differentiation, transforming growth factor (TGF)-β, IL-1β, IL-6, IL-23, IL-21 and the key transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt)[73].

Given the changes in T-cells priming, it is safe to assume that TH17-cells will also be affected by aging. Accordingly, studies in both humans and mice showed an increase in TH17 differentiation with age[75]. Autoimmune or inflammatory diseases, which are common in elderly individuals, were found to have significantly higher numbers of cells expressing IL-17 mRNA and the transfer of IL-17-expressing CD4 T-cells from old mice into RAG1$^{-/-}$ mice induce

a more severe colitis than when cells from young mice were transferred[75,76]. These results hint to the pathogenic properties of TH17-cells. Interestingly, although a general increase in mRNA encoding *IL17* was observed when TH17-cells were examined in older humans, this increase was only measurable in CD4 T cells with a naïve phenotype, at the same time, when memory cells were examined, IL-17-producing cells were also significantly increased in this CD4 T-cell subset[75]. The results suggest that CD4 cells from the elderly are already more predisposed to TH17 differentiation than CD4 cells from young individuals. Since TH17-cells share similar transcription factors and require similar cytokines for their differentiation as the IL-10-producing Type 1 Regulatory T-cells (TR1), it is possible that the IL-10 increases in elderly is due to a trans-differentiation from TH17-cells to TR1 cells.

1.3.2.2.3 T-follicular helper (TFH) cell

T-follicular helper (TFH) cells are responsible for the development and maintenance of germinal centre (GC) B-cells. They mediate the selection of high-affinity B-cell clones in the GC and thereby define memory B-cell compartments[77]. Dysregulated development or function of TFH cells results in impaired vaccine response, autoimmunity or immunodeficiency[78-80]. During the aging process, factors required for differentiation and maintenance of TFH cell become dysregulated. For instance, Inducible T-cell COStimulator (ICOS), a co-stimulatory receptor required during naïve CD4 T-cell interaction with DCs, B-cells and for the induction and maintenance of B-cell lymphoma (Bcl)-6, is reduced[81]. This reduction of ICOS expression could affect the quality of signals received by naïve CD4 T-cells, thereby limiting the number of effective/efficient differentiated TFH cells.

CD40L is important for initiating and maintaining the function of the GC response. It also plays a pivotal role in initiating class switching and somatic hypermutation of antibodies[82], implying that irregularities to the expression of CD40L could contribute to altered antibody formation and GC response following encounter with antigens. In both humans and mice, the expression of CD40L is reduced and high levels of DUSP4 have been shown to lead to the reduction of CD40L expression[83].

Cytokines such as IL-6 and IL-21, involved in promoting the generation of TFH cells via Bcl-6 induction, are increased with age[84,85]. However, this finding conflicts with other results that showed reduced TFH frequencies in elderly individuals[86]. Further investigations will be important in determining whether these cytokines are altered with age and how they affect IL-10 production in the CD4 TFH population with advancing age.

1.3.2.2.4 Regulatory T-cells (Tregs)

Regulatory T-cells (Tregs) are heterogeneous populations consisting of phenotypically distinct subsets[87] that can be characterised by their Forkhead box P3 (FoxP3) expression. Based on where these cells are generated, the markers they express of their specific function, they can be differentiated into thymus derived Tregs (tTregs) or peripheral Tregs (pTregs) for those induced in the periphery. tTregs react strongly to self-antigens and so are suppressers of autoimmunity but because these tTregs do not produce a lot of anti-inflammatory cytokines, their suppression is mainly as a result of cell-to-cell contact dependent mechanisms[88,89]. pTregs as the name implies, are generated in the periphery when foreign antigens are present in the system. These cells start off as $CD4^+$ $FoxP3^-$ T-cells which then differentiate, under the influence of cytokines such as IL-2 and TGF-β, to $FoxP3^+$ cells[88]. They do not express the transcription factors helios and neuropilin-1 but produce anti-inflammatory cytokines such as IL-10, IL-35 or TGF-β, that downregulate co-stimulatory molecules CD80/CD86 on antigen-presenting cells (APCs)[88], all of which modulates immune responses by inhibiting the differentiation of naïve T-cells or proliferation of effector T-cells.

Although data on Tregs show that their numbers in primary lymphoid organs and in the blood increase with age due to hypomethylation of CpG islands within promoter and enhancer regions[90,91], aging has more adverse effects on Tregs than on conventional T-cells, as mice data show that Tregs from old mice are less efficient in suppressing effector cells than Tregs from young mice[92]. Indeed, human *in situ* studies with skin biopsies showed that older individuals had higher percentages of Tregs with or without antigen stimulation, suggesting an increase Treg numbers in steady state in older adults[91]. This increase in Treg numbers could be a potential source of increased IL-10 production and suppression of the immune response to new antigens and vaccination in the elderly.

Apart from suppressing immune responses, Tregs from the elderly can contribute to the proinflammatory state reported in elderly individuals. A recent study highlighted the importance of Dbb1 and CUL4 associated factor (DCAF) 1 expression by Tregs in suppressing excessive ROS production. DCAF1 also prevented conventional T-cells from acquiring an aging phenotype by suppressing T-cell activation, cytokine production and the expression of an aging-related gene $p16^{Ink4a}$ [92]. In older individuals, DCAF1 expression is reduced resulting in compromised ROS regulation[92] and deteriorating Treg function. This loss of DCAF1 with age

might explain why aged Treg are insufficient in suppressing TH17 induced inflammation in the elderly.

1.3.2.2.5 Type 1 Regulatory T-cells (TR1)

TR1 cells are another subset of regulatory CD4 T-cells that do not express FoxP3 but produce suppressive cytokines to modulate immune responses. These cells were first described by Roncarolo et al., in the blood of severe combined immunodeficiency (SCID) patients and were seen to inhibit T-cells in an antigen specific manner[93,94]. To date, several cytokines such as IL-6, IL-21, IL-27 and even IL-10 are linked to the development of these FoxP3-negative ($FoxP3^-$) regulatory cells[95]. TR1 cells lack the expression of a specific lineage marker but have been shown to express LAG3, CD49b, and upregulate the suppressive molecule CTLA-4 after TCR stimulation[96,97]. They do, however, exert most of their regulatory functions by secreting high amounts of TGF-β and IL-10[97]. TR1 cells also inhibit adaptive immune activation by producing granzyme B and perforin to facilitate a protease mediated killing of myeloid APCs[98]. By activating IL-10 signalling cascade, TR1 cells upregulate immunomodulatory molecules like immunoglobulin-like transcript (ILT) 3 and ILT4 on DCs thereby inhibiting DC maturation and inducing T-cell tolerance[99]. In addition to the anti-inflammatory cytokines secreted, these cells to an extent, secrete IFNγ[94], making them resemble TH1-cells.

TR1 cells are indispensable in building a tolerance to airway allergens, as they suppress pathogenic TH2-cell development they also improve transplant tolerance[100,101]

There is a paucity of data on how these TR1 cells react in the aging process, but the increased production of IL-10 with age points to an accumulation of such high IL-10-producing cells. In a study of CD4 T-cells from aged HIV patients, increased IL-10 production was derived from virus-specific TR1-like cells. The authors also found evidence that the increased IL-10 was protective against viral replication *in vitro*[102], therefore suggesting a protective function of TR1 cells in old age, which is in line with findings linking increased IL-10 to longevity in centenarians[103,104].

In summary, aging of the immune system is associated with drastic changes in the proliferation, distribution and performance of immune cells, with increased inflammatory processes inadvertently leading to an increase in anti-inflammatory mediators such as IL-10 and compromising immunity to infection and disease.

1.4 Cytokine dysregulation and its role in aging

Cytokines coordinate immune responses to invading pathogens through development and maturation of myeloid and lymphoid cell lineages. Any alteration in circulating concentrations of cytokine will impact immune cell distribution, development and activation in response to stimuli.

A consequence of immunosenescence is the increased production of proinflammatory mediators such as TNF, IL-6, IL-1β and C-reactive protein (CRP), in the absence of an infection (sterile condition). This imbalance between pro- and anti-inflammatory cytokine describes the "inflammaging" phenotype [105], another hallmark of aging and animal as well as human studies consistently report increased activation of inflammatory pathways with age[106,107].

Apart from age-related changes that affect immune cells and ultimately increased secretion of inflammatory mediators by immune cells, other theories describe the cause of inflammaging as resulting from an accumulation of fat, particularly visceral, pericardial or intramuscular fat, which increases inflammatory mediators like IL-6 or TNF[107]. This release of cytokines from adipocytes is as a result of constant crosstalk between the immune cells embedded within the adipose tissue. Due to hypertrophy of fat cells and the resulting poor vascularisation, areas within the adipocyte become hypoxic or necrotic resulting in increased release of proinflammatory mediators[107] (Fig. 1).

The increase in age-related senescent cells, senescence-associated secretory phenotype (SASP) or skewed redox balance are some of the other pathways whose disruption is also linked to the inflammaging phenotype. For example, aging leads to an accumulation of senescent somatic cells (SSCs), but diminished chemotaxis of immune cells to these SSCs and impaired phagocytosis of such SSCs for targeted killing leads to their accumulation and subsequent generation of somatic cellular SASPs[47,108]. SASPs secrete proinflammatory factors such as IL-6, IL-1β, matrix metalloproteases (MMPs), ROS and modulate tissue development[48]. Accumulation of SSCs and the accompanying increases in SASP factor damages tissues (Fig. 1). This effect was observed in IL-10 deficient (IL-$10^{-/-}$) mice, which exhibit an aging phenotype with increased SSCs and SASP factors[109]

Beyond SASP factors, mitochondrial dysfunction and ineffective autophagy due to inflammasome activation are involved in the increase of inflammatory mediators in the elderly. In aged cells, enlarged mitochondria lose structural integrity, but these aged mitochondria do not receive a lot of damage to their membranes and are therefore less

targeted for autophagy[110]. They accumulate in cells, produce a lot of damaging ROS, that stimulates the NLR family pyrin domain containing 3 (NLRP3) component of the inflammasome, inflammatory caspase-1 is recruited and cytokines like TNF, IL-1β and IL-6 are secreted[111], thereby increasing the overall inflammatory state (Fig. 1).

Another theory attributes inflammaging to the permissive nature of the gut in elderly individuals. It is believed that bacterial products frequently permeate the leaky gut barrier and enters the bloodstream more easily, thereby eliciting an immune response[112]. Another study reported that changes to the gut microbiome with a decrease in *Bifidobacteria* population and an increase in facultative anaerobes like *Streptococci, Enterococci* or *Enterobacteria*, are associated with increased IL-6 and IL-8 with age[113].

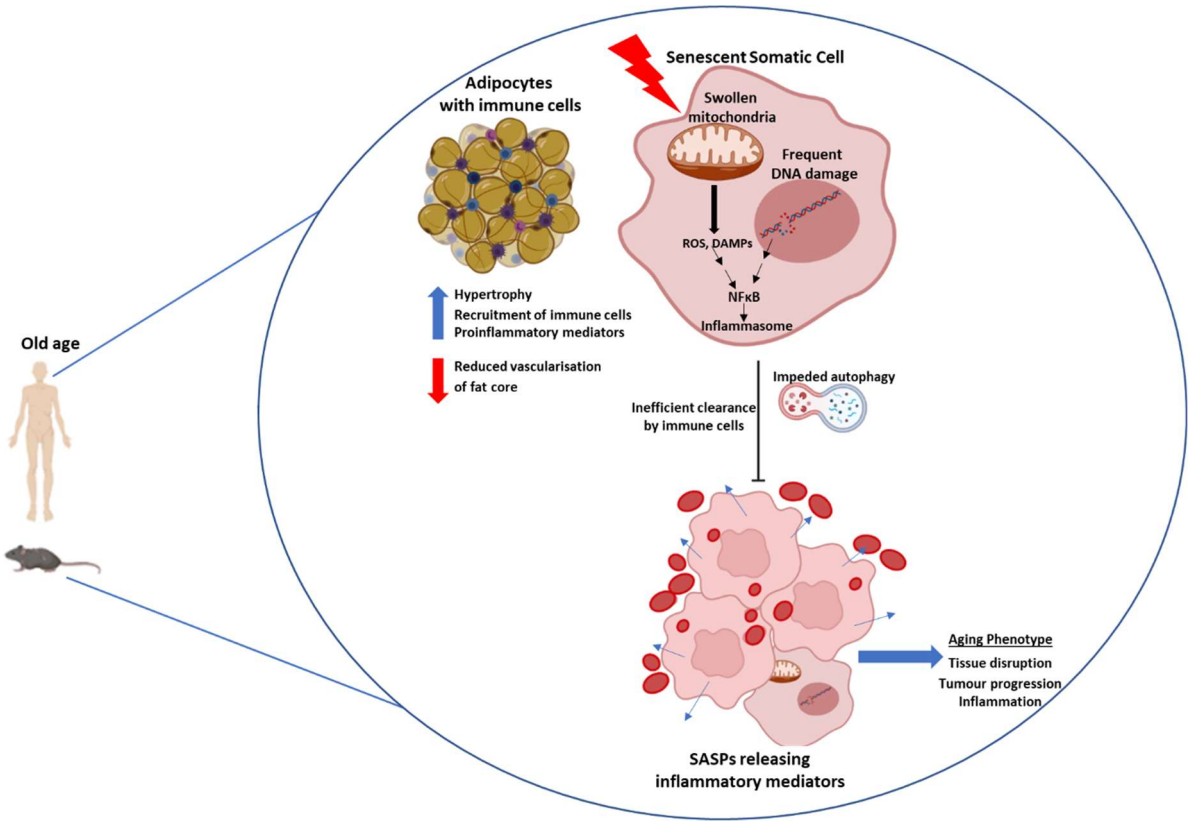

Figure 1. Factors causing or augmenting inflammaging
Increase in total body fat mass with age is results in an increased release of inflammatory mediators that then cause damage to organs in close proximity to the fat cells. Impaired autophagy, increased DNA damage, and mitochondrial dysfunctions ultimately leads to the accumulation of senescent cells that secrete factors that make up senescence-associated secretory phenotype (SASPs) and can trigger the aging phenotype and cancer
Diagram created with Biorender

A commonality between these inflammaging inducing molecular pathways is the activation of NFκB and release of cytokines, most noticeable, IL-6 cytokine whose excessive production is a predictive factor for frailty[114]. Interestingly, not only are proinflammatory cytokines elevated

with age, but there are also reports of increased anti-inflammatory species like Interleukin receptor antagonist, soluble TNF receptor 1 (sTNFRI), sTNFRII and IL-10 in elderly individuals[115,116], which are therefore considered markers for inflammaging because they increase concomitantly with proinflammatory mediators. The balance between pro- and anti-inflammatory cytokines could be used to predict healthy aging.

1.4.1 IL-10

Production of the homodimeric IL-10 is a complex process as a variety of stimuli induce its secretion from a vast number of haematopoietic and non-haematopoietic cells. For example, via signal transducer and activator of transcription factor (STAT) protein activation, cytokines such as IL-6, IL-21, IL-27, TGF- β and even IL-10 itself induce its production.

As a potent anti-inflammatory mediator, IL-10 ensures the resolution of inflammatory responses and prevents immune-related pathology by activating STAT3 upon signalling through its two-receptor complex[117]. Although STAT3 is also activated following proinflammatory cytokine stimulation, its role in IL-10 immune suppression was highlighted when STAT3 was deleted from mouse macrophages and neutrophils as these mice developed colitis-like disease[118]. IL-10 inhibits major histocompatibility class (MHC)-II expression on APCs as well as prevents DC differentiation thereby preventing effective activation of TH1 or TH2-cells. It supresses TNF and IL-6 transcription through the action of miR-187 and represses CD28 thereby affecting signalling in CD4 cells[119,120]. Notably, IL-10 acts anti-immune as seen in the colitis model where transfer of IL-10-producing TR1 cells alleviates inflammation[94].

Circulating IL-10 levels are also influenced by aging. Where some studies report increased serum IL-10 in older mice and humans[115,121], other report of a decrease in IL-10 levels in certain organs and tissue[122] that could explain the increased inflammatory state with age. Indeed, proinflammatory cytokines like IL-6, TNF and IL-1β were increased when IL-10 was diminished[123]. A likely explanation for this discrepancy in results could be due the short half-life and rapid decay of IL-10[124] which complicates its measurement.

In humans, a single nuclear polymorphism (SNP) in the *IL10* gene resulted in higher levels of secreted IL-10 that was associated with healthy aging, as centenarians with this SNP had a better aging profile and possessed more serum IL-10 in comparison to their frail counterparts[103,104]. It is however noteworthy, that disproportionately increased IL-10 levels could contribute to impaired immune responses to infections or vaccines. For example, in mice overexpressing IL-10, TB disease exacerbated and bacterial burden remained high

throughout infection[125]. IL-10 levels in serum samples of aged humans and mice are reportedly increased, but to date the cellular source of increased IL-10 in the context of aging has not been investigated.

1.4.2 IL-6

As stated above, immunosenescence results from an imbalance between inflammatory and anti-inflammatory mediators one such mediator that is increased with age is IL-6, a pleiotropic cytokine that is produced by haematopoietic cells like B-cells, macrophages, DCs and non-haematopoietic cells including fibroblasts, epithelial cells, and keratinocytes[126]. Under physiological conditions, IL-6 serum levels are extremely low, but this increases dramatically during inflammatory responses[84].

Following stimulation by a variety of external stimuli like LPS, IL-6 production is induced and its numerous functions are initiated when the heterotrimeric complex of IL-6, its receptor alpha (IL-6Rα) and the transmembrane protein gp130 is formed[127] to enable either classical signal transduction via its membrane-bound receptor or trans-signalling via its soluble receptor. This complex in turn, orchestrates the transphosphorylation and activation of downstream targets that include the Janus tyrosine kinase (JAK1, JAK2, and TYK2) family, the STAT1 and STAT3, the Src homology 2-containing tyrosine phosphatase (SHP-2)/ ERK /mitogen-activated protein kinase (MAPK) pathways[128] or the PI3K/Akt pathway[129]. Phosphorylation of STAT molecules or MAPK results in the transactivation of target genes. Whereas STAT signalling cascade is terminated in negative feedback mechanism, with the induction of suppressor of cytokine signalling (SOCS) 1 or SOCS3, ERK/MAPK and PI3K/Akt pathways are terminated by the action of dephosphorylating enzymes on their serine/threonine residues

IL-6 governs many biological functions such as metabolism, tissue regeneration, immune development, and regulation. After its synthesis is triggered, it induces a range of acute phase proteins such as CRP, fibrinogen, and serum amyloid A (SAA). Persistent SAA production leads to the deposition of fibril amyloid in organs and this causes the deterioration of said organ[130]. By stimulating receptor activator of nuclear factor kappa-B ligand (RANKL) production, IL-6 is responsible for the activation and differentiation of osteoclasts in bone marrow stomal cells[131] leading to bone resorption and osteoporosis. In the innate immune system, monocytes differentiation to macrophages instead of DCs is influenced by IL-6[132], it supresses neutrophil infiltration while supporting the infiltration of mononuclear leucocytes[133]. IL-6 also acts on

adaptive immune cells by inhibiting apoptosis, facilitating T-cell survival by maintaining Bcl-2 expression[134] and downregulating Fas ligand (FasL) expression on CD4 T-cells, as well as promoting TH-cell differentiation. For example, IL-6 upregulates the secretion of IL-4 by inducing the expression of nuclear factor of activated T cells 2 (NFATc2); the secreted IL-4 then aids the differentiation effector TH2-cells[135]. The enhancing effects of IL-6 on TH2-cell differentiation may explain the bias towards a TH2 immune response in elderly individuals and the higher frequency of Tuberculosis infection occurrence in the aged population. IL-6 indirectly diminishes TH1-cells via JAK/STAT signalling and the subsequent expression of SOCS1 which then inhibits IFNγ production[136]. IL-6 is a prerequisite for the differentiation of naïve CD4 cells to TH17-cells, it is therefore probable that IL-6 is also indirectly responsible for the increased TH17-induced chronic inflammation seen in the elderly[137]. IL-6 signalling through the JAK/STAT pathway can induce *IL21* gene expression in CD4 cells, a crucial gene for TFH cell development in GCs. Induction of the *IL21* gene aids the differentiation of activated B-cells into antibody producing plasma cells[138,139], so that continuous IL-6 production might result in autoantibody production. Attributable to its pleiotropic nature, IL-6 is also involved in the differentiation of IL-10-producing Tregs and TR1 cells by promoting the production of IL-27[95,140].

Aging is associated with elevated serum IL-6 and is thus, implicated in the pathogenesis of many age-related diseases especially those resembling chronic inflammation or autoimmune disorders. However, there are important gaps in our knowledge on the role of IL-6 in modulating CD4 T-cell subsets that can impair immune responses in elderly individuals.

1.4.3 IL-21

Produced by CD4 cells, B-cells, dendritic cells, and natural killer T (NKT) cells, IL-21 is a pleiotropic cytokine with diverse effects on a wide range of immune and non-immune cells. It is thought to increase with age as a result of increased STAT4 phosphorylation in response to IL-12[85]. Like IL-6, IL-21 also activates pathways such as the JAK/STAT, PIP3K/Akt and the ERK/MAPK pathways leading to proliferation and IFNγ secretion from NK cells[141].

IL-21 acts mostly on B-cells as evidenced by IL-21 receptor deficient mice, which had normal lymphoid population in the thymus but developed abnormalities in their B-cell function with elevated IgE and decreased IgG2b and IgG3[142]. By inducing B lymphocyte-induced maturation protein (Blimp)-1, IL-21 via JAK/STAT signalling causes B-cell terminal differentiation to plasma cells. Given its role in B-cell development and antibody production, increased IL-21 production

could exacerbate the generation of autoantibodies, as experiments in NOD mice with increased pancreatic IL-21 resulted in a type-1-diabetes-like phenotype[143]. Similarly, BXSB-Yaa mice developed severe lupus with increased IL-21 production[144]. Corresponding to mice studies, humans with systemic lupus erythematosus also have elevated IL-21 suggesting that uncontrolled IL-21 production increases the risk of developing autoimmune disorders[145]. IL-21 modulates TH1 differentiation by supressing eomesodermin, a T-box transcription factor, thereby strengthening TH2 responses[71]. IL-21 contributes to inflammatory response through its IL-21/STAT3 axis and is essential for IL-17 expression from TH17-cells. Indeed mice deficient in IL-21, its receptor-α or the nuclear receptor RORγt lacked in TH17-cell population[146]. IL-21 also activates IFNγ genes when STAT proteins bind the IFNγ response elements and augments inflammatory processes by increasing infiltrating neutrophil, NK-cells and CD8 cells *in vivo*[147], suggesting that increased secretion of IL-21 in aging amplifies the proinflammatory phenotype and increases the production of defective antibodies in the elderly. Conversely, IL-21 also has negative effects on immune cells by inhibiting DC maturation, increasing B-cell apoptosis and increasing IL-10 production in T-cells. In mice deficient of IL-21, IL-10 production was reduced while in IL-21 transgenic models, IL-10 production increased[148]. Moreover, IL-21 induction is necessary for the generation of IL-27 induced TR1 cells and simulation of TCR in the presence of IL-21 increased IL-10 production in already committed TH-cells[148]. However, our knowledge on how IL-21 influences CD4 T-cell immune responses during aging and immunosenescence is still under investigation.

1.4.4 IL-27

Belonging to the IL-12 super family, IL-27 is a heterodimeric cytokine comprising of a p28 and an Epstein-Barr virus-induced gene 3 (EBI3) subunits that can be secreted independently from one another[149]. IL-27 is mainly produced by activated macrophages and dendritic cells with one study reporting production in CD4 T-cells during infection[150].

Like IL-6, the receptor complex for IL-27 is made up of an IL-27Rα [also known as WSX-1 or type I T cell cytokine receptor (TCCR)] and a gp130 subunit, whereby expression of IL-27Rα is limited to naïve and memory B- and T-cells, resting NK cells, NKT-cells and Treg[149]. IL-27 binds to the IL-27Rα with low affinity, however, signalling begins only when gp130 is present. The binding of the cytokine to its receptor subunits results in the phosphorylation of tyrosine residues, the recruitment of JAK kinases and a subsequent phosphorylation of STAT (predominantly STAT1) proteins[149].

At its discovery, IL-27 was classed as an inflammatory cytokine due to its ability to stimulate IFNγ production in TH1-cells by upregulating Tbet and IL-12Rβ2 chain[151]. It also induces granzyme B and perforin production in CD8 T-cells in a STAT1-dependent manner[152]. Conversely, other studies have revealed its anti-inflammatory properties. For example, IL-27 is thought to attenuate TH1, TH2 and TH17 immune responses by generating a $Tbet^{+}$ Treg subset and suppressing the expression of GATA3 and RORγt, respectively[73]. It is also involved in the generation of Foxp3 negative regulatory TR1 cells[95].

Elevated systemic IL-27 levels are considered a good biomarker for early detection of cardiopulmonary failure[153]. In addition, high IL-27 levels in elderly patients correlated with a more severe disease course and longer hospital stays in COVID-19 patients, as IL-27 is thought to influence T-cell responses by upregulating inhibitory receptors such as T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) or T-cell immunoglobulin and mucin domain-containing protein (TIM) 3 and activating IL-10 production[154]. Whether IL-27 is increased with age and promotes increased IL-10 production by subsets of regulatory CD4 cells that favour the immunosenescent phenotype is still unknown.

Overall, inflammaging leads to dysregulated cytokine production. Of note here is IL-10, which has a significant inhibitory effect on T-cells. However, the cellular source of this cytokine in elderly individuals has not been carefully studied, and its interaction with other cytokines during aging is not known. Furthermore, how this dysregulated cytokine network affects the immune response after infection has also not been investigated.

1.5 Aims

Previous work describes how changes to the immune system with age elicits an underlying proinflammatory state skewing cytokine production. As a consequence of the age-related increase in proinflammatory mediators, anti-inflammatory cytokines such as IL-10 are reported to also increase with age. Although IL-10 most likely has a detrimental effect on the protective immune response in the elderly, deciphering the complex interplay between cytokines that induce IL-10 and its cellular sources during aging remains a major challenge.

The work in this thesis identified CD4 T-cells as a potential source for the increased IL-10 production in old age. CD4 T-cells are at the core of the adaptive immune response and produce different helper phenotype subsets. It is unclear which subset of CD4 T-cells are responsible for the increased IL-10 production in aging. Therefore, the work in this thesis aimed to:

- Characterise the CD4 T-cell subset(s) responsible for increased IL-10 with age
- Identify other cytokine pathways involved in modulating IL-10 production with age
- Investigate whether these IL-10-producing cells also increased in the lungs of old *Mtb*-infected mice and can altering the frequency of these IL-10-producing cells improve immune response

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Consumables and supplies

Unless stated otherwise, the disposable plastics used were obtained from Sarstedt (Nümbrecht, Germany), Sigma-Aldrich® (München, Germany) and Merck Millipore (Darmstadt, Germany).

2.1.2 Apparatus

Table 2: Apparatus used for the experimental procedures

Device	Supplier
Canto II	BD USA
Fortessa I & II	BD, USA
Lightcycler 480	Roche, Switzerland
LSRII	BD, USA
Centrifuges	Hettich Lab Technology, Germany
TECAN reader infinite 200	Tecan Trading AG, Switzerland
Sterile hood	Thermo Fisher Scientific, USA
Coulter counter	Beckmann, USA
V-cell counter	Beckmann, USA
Microscope	Olympus corporation, Japan
MSD reader	Mesoscale, USA
Thermocycler	Bio-Rad Laboratories GmbH, USA
FACS aria sorting	BD, USA
Vortex mixer	Scientific Industries Inc., USA
Water bath	Gesellschaft für Labortechnik GmbH, Germany

2.1.3 Antibodies

Table 3: Antibodies for flow cytometry

Antibody	Clone	Isotype	Fluorochrome	Concentration [µg/mL]	Supplier
CD3ε	145-2C11	Ar Ham IgG	Purified	5	BioLegend
CD4	RM 4-5	Rat IgG_{2a}, κ	BUV395	0.67	BD
CD4	RM 4-5	Rat IgG_{2b}, κ	V450	0.16	BD
CD4	RM 4-5	Rat IgG_{2a}	V500	0.31	BD
CD8a	53-6.7	Rat IgG_{2a}, κ	BUV395	0.67	BD
CD28	37.51	Syrian Ham IgG	Purified	5	BioLegend
CD44	IM7	Rat IgG_{2b}, κ	FITC	0.78	BioLegend
CD62L	MEL-14	Rat IgG_{2a}, κ	PE	0.31	BD
CD90.2	53-2.1	Rat IgG_{2a}	APC-eFlour780	0.16	eBioscience
CXCR5	L138D7	Rat IgG_{2b}, κ	PE-Cy7	2.50	BioLegend
CXCR5	L138D7	Rat IgG_{2b}, κ	BV421	1.33	BioLegend

Antibody	Clone	Isotype	Fluorochrome	Concentration [µg/mL]	Supplier
CXCR5	L138D7	Rat IgG_{2b}, κ	BV650	1.00	BioLegend
EGR2	erongr2	Rat IgG_{2a}, κ	APC	2.00	eBioscience
2° antibody		Goat IgG $F(ab')_2$	Alexa Flour 647	0.40	abcam
FoxP3	FJK-16s	Rat IgG_{2a}	eFlour450	1.33	eBioscience
FoxP3	FJK-16s	Rat IgG_{2a}	FITC	1.33	eBioscience
FoxP3	FJK-16s	Rat IgG_{2a}	PE-Cy7	1.00	eBioscience
GP130	KGP130	Rat $IgG_{2a,}$ κ	APC	1.33	eBioscience
IFNγ	XMG1.2	Rat IgG_1	PE	2.00	BioLegend
IFNγ	XMG1.2	Rat IgG_1	BV650	1.33	BD
IFNγ	XMG1.2	Rat IgG_1	APC	0.63	BioLegend
IFNγ	XMG1.2	Rat IgG_1	PE-Cy7	1.00	BioLegend
IL-2	JES6-5H4	Rat IgG_{2b}	APC	1.25	BD
IL-6R	D7715A7	Rat IgG_{2b}, κ	PE	2.00	BioLegend
IL-10	JES5-16E3	Rat IgG_{2b}	PE	2.00	BioLegend
IL-17A	eBio17B7	Rat IgG_{2a}, κ	PerCP-Cy5.5	0.63	eBioscience
IL-27	B02P6E6	Rat IgG_{2a}, κ	Biotin	0.40	BioLegend
LAG3	C9B7W	Rat IgG_1, κ	PerCP-Cy5.5	2.00	BioLegend
LAG3	C9B7W	Rat IgG_1, κ	APC	2.00	eBioscience
LAG3	C9B7W	Rat IgG_1, κ	PE-Cy7	2.00	eBioscience
LAG3	C9B7W	Rat IgG_1, κ	BV510	2.00	BD
cMAF	sym0F1	Mouse IgG_{2b}, κ	PerCP-eFlour710	1.50	eBioscience
PD-1	RMP1-30	Rat IgG_{2b}, κ	PE-Cy7	1.25	BioLegend
PD-1	29F.1A12	Rat IgG_{2a}, κ	PerCP-Cy5.5	2.00	BioLegend
pSTAT1	D4A7	Rabbit IgG_1		5	abcam
pSTAT3	D3A7	Rabbit IgG_1		5	abcam
TCR-β chain	H57-597	Ar Ham IgG_2, λ1	BV711	0.67	BD
TNF-α	MP6-XT22	Rat IgG_1	PE-Cy7	0.16	BioLegend

Antibody	Catalogue Number	Isotype	Concentration [µg/mL]	Supplier
IL-21R Fc Chimera	596-MR	Mouse IL-21 subunit/Human IgG_1	2.00	R&D systems

2.1.4 Buffers, Media, & Reagents

Table 4: Solutions

Solutions & reagents	Composition	Supplier
Aqua destillata (ddwater)		Research centre Borstel, Germany
Aqua B. Braun		B|Braun, Melsungen, Germany
Agarose		PEQLAB, Germany
Brefeldin A		BioLegend, USA

Solutions & reagents	Composition	Supplier
Blocking buffer (v/v)	25 µg/mL purified anti-CD16/CD32 1% Hamster serum 1% Mouse serum 1% Rat serum Flow cytometry buffer (v/v)	BioLegend (clone 2.4G2) Jackson Immo Research, USA PAA, UK PAA, UK
Digestion medium (Lung)	10% FCS 1% (2mM) L-Glutamine 1% Penicillin/Streptomycin 1mM Sodium pyruvate RPMI medium 0.7 mg/mL collagenase A 30 µg/mL DNAse I	PAA, UK Biochrom AG, Germany Biochrom AG, Germany Biochrom AG, Germany Biochrom AG, Germany Roche, Switzerland Sigma-Aldrich, USA
Dulbecco's complete medium (DMEM)	DMEM 10% FCS 1% (2mM) L-Glutamine 1% Penicillin/Streptomycin	Gibco®, USA PAA, UK Biochrom AG, Germany Biochrom AG, Germany
ELISA stop solution	1M H_2SO_4	
ELISA wash buffer	0.05% Tween-20 1x PBS	Roth Germany Gibco®, USA
Fetal Calf Serum (FCS)		PAA, UK
Florescence Associated Cell Sorting (FACS) buffer	1x PBS 1% FCS	Gibco®, USA PAA, UK
Flow cytometry buffer (v/v)	1x PBS 0,1% NaN_3 3% FCS	Roth, Germany Roth, Germany Roth, Germany
FoxP3 staining & fixation buffer		eBioscience, USA
HEPES		Gibco®, USA
IMDM complete medium	10% FCS 1% (2mM) L-Glutamine 1%Penicillin/Streptomycin IMDM (Iscove's modified Dulbecco's) medium	PAA, UK Biochrom AG, Germany Biochrom AG, Germany Gibco®, USA
Magnetic associated cell sorting (MACS) Buffer	PBS (without Ca^{2+} and Mg^{2+}) 2 mM EDTA 0.5 % BSA	Gibco®, USA Roth, Germany PAA, UK
Monensin		BioLegend
10x Phosphate-buffered saline (PBS) without Ca/Mg		Gibco®, USA

Solutions & reagents	Composition	Supplier
Red cell lysis buffer	156 mM NH_4Cl 0.13 mM EDTA 12 mM $NaHCO_3$ H_2O	Merck, Germany Roth, Germany Merck, Germany Braun, Germany
Resuspension medium (Differentiation medium)	RPMI 1640 with HEPES 10 % FCS 1 Penicillin/Streptomycin 1 % (2mM) L-Glutamine 50 µM 2-Mecptoethanol GM-CSF	Gibco®, USA PAA, UK Biochrom AG, Germany Biochrom AG, Germany Milipore, USA BioLegend
RPMI	10% FCS 1% (2mM) L-Glutamine 1% Penicillin/Streptomycin	PAA, UK Gibco®, USA Biochrom AG, Germany
RNAse AWAY		Molecular Bioproducts, USA
TRIzol reagent		Thermofisher Scientific, USA
Wash buffer- Hanks balanced salts (HBSS)	3% FCS HBSS	PAA, UK Gibco®, USA

2.1.5 Kits

Table 5: Kits for qRT-PCRs and ELISAs

Kits	Supplier
IL-6 receptor alpha ELISA kit	R & D Systems Biotechne, USA
MSD multiplex assay ELISA kit	Mesoscale, USA
RNeasy Mini kit	Qiagen, Netherland
sgp130 ELISA kit	R & D Systems Biotechne, USA
Reverse transcription kit	E6300s BioLabs, USA

2.1.6 Enzymes, Primers and Cytokines

Table 6: qRT-PCR primers and cytokines

Enzyme	Supplier
Superscript II Reverse Transcriptase (10000 U)	Invitrogen, USA
Primers	**Sequence**
IL-6 Primer	Forward- 5-AACGATGATGCACTTGCAGA-3′ Reverse- 5-TGGTACTCCAGAAGACCAGAGG-3′
IL-10 Primer	Forward- 5′ -GCTCTTACTGACTGGCATGAG-3′ Reverse- 5′ -CGCAGCTCTAGGAGCATGTG-3′
S-14 Primers	Forward- 5-GAGGAGTCTGGAGACGACGA-3′ Reverse- 5-TGGCAGACACCAAACACATT-3′
Recombinant cytokines	**Supplier**
IL-6	BioLegend, USA
IL-27	R & D Systems Biotechne, USA

2.1.7 Bacterial strain and culture

Mtb H37Rv was cultured at 37°C in a Middlebrook 7H9 broth supplemented with 10% oleicacid-albumin-dextrose-catalase (OADC), 0.05% Tween 80 and 0.5% glycerol untill the mid-logarithmic phase (2.5 x 10^8 bacteria/mL) by Silvia Maaß (RCB) and stored at -80°C for further use.

2.1.8 Experimental animals

Mice used in all experiments were approved of by the *"Ministerium für Landwirschaft Umwelt und ländliche Räume des Landes Schleswig-Holsteins (Kiel, Germany)"* ethics committee for animal Experiments under the reference number (Ref. №): V241 – 51057/2017 (109-8/17) and by the "*Intuitional Animal Care and Use Committee*" at the Cincinnati Children's Hospital Research foundation (Ref. №: IACUC 2016-0087).

In the present thesis, young mice are classified as 4 weeks (Wks) old — ≤ 4 months (mths) old while aged mice are classified as ≥ 12 months old

Animals were bred and kept in pathogen-free conditions, while the animals to be infected were housed in biosafety level 3 (BSL-3) containment areas in individually ventilated cages (IVCs)

The mice used in all experiments were of a C57BL/6 genetic background (Table 7).

Table 7: Mouse strains and suppliers

Mice strains	Supplier
C57BL/6	National Institute of Health (NIH), USA or in house breeding at the Research Centre Borstel (RCB), Germany
CD19 cre IL-$10^{f/f}$	Rudolf Manz (Institute for Systemic Inflammation Research-ISEF, Universität zu Lübeck), Germany
$FoxP3^{DTR}$	David Hildeman (Division of Immunobiology, Cincinnati Children´s Hospital Medical Centre, Cincinnati), USA
IL-$6^{-/-}$	David Hildeman (Division of Immunobiology, Cincinnati Children´s Hospital Medical Centre, Cincinnati), USA
IL-6Rα$^{f/f}$ CD4 $CreER^{T2}$	David Hildeman (Division of Immunobiology, Cincinnati Children´s Hospital Medical Centre, Cincinnati), USA
P25KtkC45Z	In house breeding at the Research Centre Borstel (RCB), Germany
PEPCU-sgp130 FC	Christoph Garbers (Otto-von-Guericke-Universität Magdeburg Institut für Pathologie) Magdeburg
$STAT3^{f/f}$ CD4 $CreER^{T2}$	David Hildeman (Division of Immunobiology, Cincinnati Children´s Hospital Medical Centre, Cincinnati), USA
IL-$21^{-/-}$	David Hildeman (Division of Immunobiology, Cincinnati Children´s Hospital Medical Centre, Cincinnati), USA
IL-27Rα$^{-/-}$	In house breeding at the Research Centre Borstel (RCB), Germany

2.2 Methods

2.2.1 *in vivo* animal experiments

2.2.1.1 Aerosol infection

To determine the concentration of the H37Rv aliquot used, a thawed aliquot was mixed 10 times using a 26G-needle (Braun) to thoroughly break-up any aggregates and then diluted to a concentration of 2.5 x 10^6 bacteria in 6 mL 0.05% Tween80 (Roth) ddH_2O (aqua B.Braun). A 10-fold serial dilution is then performed with 0.5 mL of this solution and 100 µL of the serial dilutions are plated out on 7H10 agar plates supplemented with bovine serum, followed by incubation at 37°C and 5% CO_2 (HERAcell 150i CO2 incubator, Thermo Scientific) for 21 days. The remaining 5.5 mL solution was drawn-up into a 10 mL syringe with an 18G needle, placed in a transport box, transferred to the aerosol machine (Modell 099C A4224, Glas-Col®, Terre Haute, USA) and used to infect the mice with 100 CFU of the *Mtb* strain H37Rv. To achieve this, the mice were placed in the aerosol machine in individual aerosol chambers (Marine & Industrie Technik, Hamburg, Germany) and the 5.5 mL bacterial solution was then injected into the nebuliser (Glas-Col®). The programme was then run with a flow rate for the main air flow at 60 CFH (cubic feet per hour, = 1.68 m^3/h) and with a flow rate of 11 CFH (0.28 m^3/h) for the compressed air to nebulize the infectious solution. For the infection, 15 minutes of warm-up, 40 minutes of nebulisation, another 40 minutes of cloud decay (aspiration) and finally 15 minutes of decontamination were set. After which, the mice were removed and transferred back into IVCs. One day after infection, 2 mL of 0.05% Tween80 (Roth) ddH_2O (aqua B.Braun) lung homogenates from 4 infected mice were plated out on 7H10 agar plates supplemented with 10% bovine serum and cultured at 37°C 5% CO_2 for 21 days to verify the dose of infection.

2.2.1.2 Scoring

The Morton and Griffith scoring system[155] was used to assess the disease severity. Mice were examined regularly and scored on a scale of 1—5 based on their weight, appearance and behaviour (Table 8). Mice with a score of 3.5 were classified as moribund and therefore euthanised and processed before the originally scheduled end of experiment date.

Table 8: Scoring system

Score	Activity	Weight loss	Behaviour	General condition
1	Very active	Increase or no change	Normal. e.g. Tail movement, curiosity	Shiny clean coat, clear eyes
2	Active	< 10 %	Slight deviation from normal	Increase or decrease in self-grooming
3	Less active	10 – 20 %	Limited motor skills	Self-neglect, dull coat increased twitching
4	Sluggish movements	20 – 30 %	Dirty fur, sticky or moist body openings, cloudy eyes	Self-isolation, lethargy, coordination disorder
5	Lethargic	> 30 %	Cramping, paralysis, animal is cold to touch	Painful noises when held, auto-aggression

2.2.1.3 Harvesting of organs from Mtb infected mice for CFU counts and Flow cytometry analysis

At indicated timepoints or in the case of moribund mice, the bacterial load as well as the immunological responses in various organs of the infected mice were determined. Using standard procedures approved under animal welfare regulations, the mice were killed in a CO_2 chamber. Euthanised mice were fixed dorsally recumbent on a dissection board. For genotyping of the mice, a piece of tail was cut off, placed in a 1.5 mL Eppendorf tube and stored at -80°C. Using a pair of scissors and forceps, an incision was made along the entire length of the ventrum to expose the abdominal cavity, the viscera were pushed aside and approximately 1 mL of blood was then drawn from the inferior vena cava and transferred into serum separator tubes (KABE LABORTECHNIK GmbH). The serum separator tubes were then centrifuged for at 6000 rpm and 4°C for 10 minutes. The serum was stored in separator tubes at -80°C for further analysis.

One group of mice was used to determine the bacterial load, while another group was used to analyse the immunological responses to *Mtb* infection. To determine the bacterial burden in the Lungs, the rib cage was cut open, whole Lung tissue was removed, weighed then divided up for use in different analysis. The left Lung lobe was weighed again and placed in a tube containing 3 mL PBS mixed with protease inhibitor (Roche) and homogenised, while for the determination of bacterial load in spleen, half of the spleen was put into a tube containing 8 mL 0.05% Tween80 (Roth) ddH_2O (aqua B.Braun) for homogenisation.

To prepare lung single cell suspensions, the inferior vena cava was first severed and the lung perfused with 5 mL of 1x PBS (37°C) through the right ventricle with a 25G needle (BD) until all the blood was flushed out. After perfusion, the Lung was excised and placed in a petri dish with 1 mL of 1x PBS. Using scalpels (Paragon®, Sheffield, UK), the lung was cut up into small pieces and transferred to a 50 mL Falcon tube filled 10 mL digestion medium. The lungs were then digested in a water bath shaker (TW20, Julabo, Seelbach, Germany) at 37 °C for 2 hours. The spleen was also removed from the mice and transferred to 15 mL flask tubes filled with 5 mL HBSS wash buffer.

2.2.1.4 Determination of bacterial burden in the lungs and the spleen

After whole lung tissue was collected and weighed, the lung was then divided up into its different lobes. The left lung lobes were transferred into FastPrep tubes (Sarstedt) containing 6 ceramic beads (MP Biomedicals) and 3 mL of 1x PBS + protease inhibitor (Roche), while spleens were also transferred into FastPrep tubes with 5 ceramic beads (MP Biomedicals) and 8 mL of 0.05% Tween80 (Roth) ddH_2O (aqua B.Braun). The organs were subsequently homogenized with the FastPrep®-24 homogenizer (MP Biomedicals). From the homogenate, 0.5 mL was taken and a 10-fold serial dilution in 0.05% Tween80 ddH_2O was performed. A volume of 0.1 mL of each dilution was plated out on a Middlebrook 7H10 agar plate and incubated at 37°C and 5% CO_2 (HERAcell 150i CO2 incubator, Thermo Scientific). Colony forming units (CFU) were counted at 21-, 42-, 49-, 72- and 84-days post infection. The bacterial burden was then calculated based on CFU and organ weight.

$$Bacterial\ load = vol\ homogenate \times CFU.Nr \times n\ dilution^{10} \left(\frac{total\ organ\ weight}{organ\ weight\ plated\ on\ agar}\right)$$

2.2.1.5 Single cell suspension for Flow cytometry

MHC II tetramer I-A^b $ESAT6_{(4\text{-}17)}$ APC (1.6 mg/mL, QQWNFAGIEAAASA) was purchased from the NIH Tetramer Core Facility at Emory University Atlanta, USA, while the $ESAT\text{-}6_{1\text{-}20}$ peptide was a gift from Rainer Bartels (RBC).

2.2.1.5.1 Lung

After lung digest, the tissue was filtered through a 100 µm sieve (Corning®) into a 50 mL Falcon tube using a 5 mL syringe plunge and HBSS wash buffer. The resulting single cell suspensions were centrifuged (1200 rpm, 10 minutes, 4°C.) and the supernatant was discarded. The cells were briefly resuspended (20—30 secs) in 3 mL red cell lysis buffer and immediately filled to 30 mL with wash buffer to stop the reaction. The cells were again centrifuged (1200 rpm,

10 minutes min, 4°C.) and the supernatant discarded. The cells were then resuspended in 2—5 mL of IMDM complete medium (Iscove's medium) and the cell number was determined. After counting, a total of $1 \times 10^6 - 2 \times 10^6$ cells were seeded in 96-well flat-bottom plates (Costar®, Corning Incorporated), polyclonally stimulated for 5 hours using anti-CD3/CD28 antibodies (5 µL/mL in PBS), or antigen-specific stimulated with STCF (10 µg/ml in IMDM) for 1 hour or ESAT-$6_{1\text{-}20}$ peptide (5 µg/mL in IMDM) for 1 hour 15 minutes and stained for flow cytometry (see section 2.2.2.2.4 below).

2.2.1.5.2 Spleen

Spleens were filtered through a 100 µm sieve (Corning®) with the aid of a 5 mL syringe plunge and HBSS wash buffer into a 50 mL Falcon tube. The single cells were then centrifuged (1200 rpm, 10 minutes, 4°C.) and the supernatant discarded. Erythrocytes were lysed by adding 5 mL of red cell lysis buffer for 2 minutes. The reaction was stopped by filling the tube to 30 mL with wash buffer and centrifuging (1200 rpm, 10 minutes, 4°C.). These cells were subsequently resuspended in 2 mL of IMDM complete medium and counted. After counting, a total of 2 x 10^6 cells was seeded into 96-well flat-bottom plates (Costar®, Corning Incorporated), polyclonally stimulated using anti-CD3/CD28 antibodies (5 µL/mL in PBS) for 5 hours or STCF (10 µg/ml in IMDM) for 1 hour or ESAT-$6_{1\text{-}20}$ peptide (5 µg/mL in IMDM) for 1 hour 15 minutes and stained for flow cytometry (see section 2.2.2.2.4 below).

2.2.1.5.3 Cell counts

An automated Vi-CELL® counter was used to calculate the number of cells recovered from the lung or spleen. Resuspended cells were diluted 1:20 in FACS buffer to a final volume of 0.5 mL in cell counter tubes (Beckmann Coulter, Miami). After counting, between $1 \times 10^6 - 2 \times 10^6$ cells were seeded into wells for flow cytometry experiments.

2.2.2 *in vitro* animal experiments

2.2.2.1 Harvesting Organs for quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Using standard procedures approved under animal welfare practices, young mice or aged mice were sacrificed. Euthanised mice were fixed dorsally recumbent on a dissection board. For real-time quantitative polymerase chain reaction (qRT-PCR) the spleen, lymph nodes, liver, gut, brown adipose tissue, epididymal and inguinal white adipose tissue were removed. All organs apart from the gut were immediately flash frozen in liquid nitrogen then stored at

-80°C. Using a 5 mL syringe, the contents of the gut were flushed out with saline before being stored at -80°C. For RNA extraction, organs or tissue were transferred into 2 mL Eppendorf tubes filled with 1.5 mL TRIzol (ThermoFisher Scientific) and a steel bead for homogenisation. RNA extraction was performed using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and quantified via nanodrop. 2 µg of DNase-treated RNA was then reverse transcribed to cDNA (BioLabs) according to the manufacture's protocol. The cDNA was analysed using a Sybr green (Roche) based qRT-PCR to detect mRNA expression of *IL10* and *IL6* in young and old mice. A master mix containing 5 µL LightCycler® 480 SYBR Green I Master Mix, 20 µM forward and reverse primer and 2.5 µL RNase free water was prepared and 8 µL was pipetted into a LightCycler® 96-well plate (Roche) and mixed well with 2 µL cDNA. A serially diluted internal standard (1:5, 1:10, 1:20, etc) as well as a negative control of water was pipetted into the LightCycler® 96-well plate (Roche) and measured on the LightCycler® 480. Analysis was performed with the LightCycler® 480 software (LCS480 1.5.0.39).

2.2.2.2 Flow cytometric experiments

Spleen tissue was filtered through a 100 µm sieve (Corning®) using a 5 mL syringe plunge being and HBSS wash buffer into a 50 mL Falcon tube. The single cells were centrifuged (1200 rpm, 10 minutes, 4°C.) and the supernatant discarded. Erythrocytes were lysed in 5 mL red cell lysis buffer for 2 minutes and the reaction was stopped by filling the tube to 30 mL with wash buffer and centrifuging (1200 rpm, 10 minutes, 4°C.). Depending on further analyses, the cells were resuspended in either 2 mL MACS or IMDM buffer, counted using a Neubauer counting chamber or a coulter counter (Beckman) and adjusted to $2 \times 10^6/mL$.

$$\text{Neubauer Chamber: } \left(\frac{count1+count2}{2}\right) \times dilut.\,fact. \times 10^4 = \frac{cells}{mL}$$

$$\text{Coulter Counter method: } (count\ 1 + count\ 2) \times 1000 = \frac{cell}{mL}$$

2.2.2.2.1 Magnetic Activated Cell Sorting (MACS):

POSITIVE SELECTION CD4 (L3T4 Milteny Biotec): - here the CD4 T-cells are magnetically labelled and held in the column. The unlabelled cells drain out with the flow through and when the column is removed from the magnet field, the labelled CD4 cells can be collected.

Cells were centrifuged at 300 x g for 10 minutes, the supernatant was discarded and 90 µL of MACS buffer per 1 x 10^7 cells was used to resuspend the cells. 10 µL CD4 microbeads for magnetic labelling were then added to the resuspended cells and incubated for 15 minutes.

After magnetic labelling, 10^7 cells were washed in 1 mL buffer at 300 x g for 10 minutes. A maximum of 10^8 cells were resuspended with 500 µL MACS buffer and then transferred to LS columns in a magnetic field that had been pre-washed with 3 mL MACS buffer. After separation, cells were resuspended in sort buffer (1 x PBS + 1% FCS) and sorted into different CD4 T-cell populations on the FACS Aria (BD).

NEGATIVE SELECTION CD4 (**130-104-454 Milteny Biotec**): - the difference to positive selection is that all lymphocytes other than CD4 T-cells are magnetically labelled and are held back in the magnetic field while the unlabeled CD4 cells flow through the column

Cells were centrifuged at 1500 rpm for 5 minutes at 4°C, supernatant was discarded and for every 1 x 10^7 cells, 40 µL of MACS buffer was used to resuspend the cells. 10 µL of a biotin antibody cocktail was added to the resuspended cells and incubated for 5 minutes at 4°C. Next, 30 µL of buffer per 10^7 total cells was added, followed by 20 µL of streptavidin-conjugated MicroBeads per 10^7 total cells. This mixture was further incubated for 10 minutes at 4°C and then transferred to LS columns in a magnetic field that had been pre-washed with 3 mL MACS buffer to separate CD4 cells from the other lymphocytes. After separation, enriched CD4 T-cells were washed in MACS buffer at 1500 rpm for 5 minutes at 4°C. and resuspended in sort buffer (1 x PBS + 1% FBS) and sorted into different CD4 T-cell populations on the FACS Aria (BD).

The LS column was removed from the magnetic field, placed onto a new 15 mL Falcon tube and the plunge used to flush out all other magnetically labelled lymphocytes. In the proliferation assay experiments, these magnetically labelled cells were irradiated and served as APCs in the experiment.

2.2.2.2.2 Phorbol 12-myristate 13-acetate and ionomycin stimulation

In 96-well flat-bottomed plates, 30µL of 2 x 10^6 lymphocyte cells were stimulated in a 30 µL mixture of 25 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin (PMA/Iono- Sigma Aldrich) for 1 hour at 37°C and 5% CO_2 (HERAcell 150i CO2 incubator, Thermo Scientific). After the hour, a 20 µL mixture of a 5-fold concentrated Monensin/Brefeldin A (Mone/Bref A- BioLegend) was added to each well and incubated for a further 4 hours at 37°C and 5% CO_2.

2.2.2.2.3 Lipopolysaccharide (LPS) Stimulation

Single cell suspension from spleen cells were obtained and 2 x 10^6 splenic lymphocyte cells were seeded in a 96-well flat-bottomed plate and stimulated with 1µg/mL LPS (Sigma Aldrich) for 24 hrs. 4 hours before the end of the incubation period, a mixture of Brefeldin A was added to the wells to block protein transport. For mock-stimulation, a buffer without LPS was added to the cells similar to stimulation conditions. Cells were incubated at 37°C and 5% CO_2 (HERAcell 150i CO2 incubator, Thermo Scientific). At the end of the incubation period, cells were spun down (1500 rpm, 5 minutes, 4°C), supernatant was collected and stored at -80°C and cells were stained for intracellular flow cytometry

2.2.2.2.4 Florescent antibody labelling: surface and intracellular stains

At the end of stimulations, cells were washed twice in a final volume of 200 µL FACS buffer (1200 rpm, 10 minutes, 4°C) and blocked with 20 µL of blocking buffer for 20 minutes at 4°C. After the blocking step, surface epitopes were stained with 80µL of a cocktail of fluorescently labelled antibodies for 45 minutes at 4°C. Thereafter, cells were washed twice in a final volume of 200 µL FACS buffer (1200 rpm, 10 minutes, 4°C) and were then fixed in 100µL methanol-free 2% PFA or a 1-fold solution of FoxP3 fixation buffer (eBioscience) for 45 minutes at 4°C. After fixation, cells were washed twice in a final volume of 200 µL FACS buffer (1200 rpm, 10 minutes, 4°C), permeabilised with 100 µL permeabilization buffer (eBioscience) for another 45 min at 4°C. Following one wash step in a final volume of 200µL permeabilization buffer, cells were labelled with fluorescently antibodies for another 45 min at 4°C to detect intracellular epitopes. After this labelling step, cells were washed twice in permeabilization buffer (1200 rpm, 10 minutes, 4°C; final volume 200 µL) and then resuspended in 200 µL FACS buffer for measurement on a flow cytometer.

2.2.2.2.5 pSTAT stimulation and florescent labelling

1 x 10^6 splenic lymphocyte cells were stimulated with 100µL 20 ng/mL murine IL-6 cytokine or hyper IL-6 (HyIL-6) for 30 minutes at 37°C and 5% CO_2 (HERAcell 150i CO2 incubator, Thermo Scientific). After stimulation cells were fixed with 2% methanol-free PFA (Thermo Scientific) for 10 minutes at 37°C and 5% CO_2. This was followed by permeabilization of the cells with 100 µL of 90% methanol (Roth) for 30 minutes on ice. After permeabilization, cells were washed twice in a final volume of 200µL FACS buffer (1200 rpm, 10 minutes, 4°C), blocked with 50µL Fcγ-receptor block (BioLegend) for 30 minutes and then labelled with primary antibodies to phosphorylated STAT (pSTAT) proteins for 45 mins at 4°C. After primary antibody

labelling, cells were washed once in a final volume of 200µL FACS buffer (1200 rpm, 10 minutes, 4°C) and stained with fluorescently labelled CD4 surface epitope antibodies and pSTAT secondary antibodies for 45 minutes. At the end of the labelling, cells were washed again in a final volume of 200 µL FACS buffer (1200 rpm, 10 minutes, 4°C) and resuspended in 200 µL FACS buffer for flow cytometry measurement.

2.2.2.2.6 Inducible Knockdown assays

Using a 23G needle, mice were injected intraperitoneally for 4 days (IL-6 receptor α knockdown) or for 5 days (STAT3 knockdown) with 100 µL of 20 mg/mL tamoxifen (Sigma Aldrich) dissolved in corn oil (Sigma Aldrich).

Firstly, Tamoxifen was weighed into a 15 mL Falcon tube under a sterile bench, then a solution of 100% ethanol and corn oil in a 1:10 ratio was prepared. This ethanol/corn oil mixture was then added to the weighed-out tamoxifen and vortexed vigorously to disrupt the pellet. At room temperature, the tamoxifen mixture was covered with aluminium foil and placed on a shaker overnight or until the pellet was completely dissolved. Mice were then injected with this solution for either 4- or 5-days and sacrificed for flow cytometric analysis exactly one week after the first injection dose.

2.2.2.2.7 Proliferation assay (irradiation method)

After negative magnetic cell separation, cells were fluorescently labelled for sorting on the FACS Aria (BD) into Effector T-cells (Teffs), IL-10-producing TFH and TR1 cells and Tregs. After sorting, cells are washed in 1-fold concentrated PBS and reconstituted in 1 mL PBS for labelling.

Teffs were counted and labelled with CellTrace™ violet (C34557; ThermoFisher). Prior to use, a 5mM CellTrace™ stock solution was prepared by adding DMSO (20µL) to one vial of CellTrace™ reagent (Component A) and mixing well to create the stock solution. To each millilitre of Teffs suspension, 1 µL of CellTrace™ stock solution was added to make the final working concentration 5 µM and this mixture was then incubated at 37°C for 20 minutes. After incubation, the labelled cells were washed with five times the volume of the initial culture medium (1500 rpm, 5 minutes, 4 °C) and incubated for 5 minutes to remove excess dye. After CellTrace™ labelling of effector T-cells, irradiated APCs, and the sorted regulatory T-cells (IL-10-producing TFH, TR1 and Tregs) were added together in a ratio of 8:1:1: (APCs : Teff : IL-10-producing TFH, TR1 or Tregs) and stimulated with anti-CD3/CD28 (0.9 µg/mL) for 72 hours.

To obtain APCs, CD4 MACS (130-104-454 kit) was performed on a single cell suspension from the spleen. All cells remaining on the LS column after negative selection were classified as APCs. After recovery of the APCs, the APCs (16 x 10^6/mL) were irradiated at 3000 rad.

- Add CellTrace™ violet (C34557; ThermoFisher) to Teff
- Sort cells into IL-10-producing TFH, TR1 and Tregs
- Teffs were obtained from young FoxP3^{DTR} mice while the regulatory T-cells were from old FoxP3^{DTR} mice

2.2.2.2.8 Proliferation assay (antigen presentation by dendritic cells)

Using a 26 G needle (BD) and a 5 mL syringe (BD), the bone marrow is flushed out of the femur of euthanised mice with wash buffer into a petri dish. For dendritic cell (DCs) differentiation, the bone marrow medium was centrifuged at 1200 rpm and 4 °C for 10 minutes, then the cells were resuspended in 2 mL of differentiation (resuspension) medium and counted in a Neubauer chamber. 5 x 10^6 cells were seeded into new petri dishes, and 10 mL of differentiation medium was added to each petri dish for incubation at 37 °C and 5% CO_2. The differentiating medium of the bone marrow derived DCs (BMDC) were replenished with 10 mL differentiation medium on day 3. On day 6, 10 mL of medium with BMDC was transferred into a 50 mL Falcon tube and centrifuged for 10 minutes at 1200rpm and 4°C. The supernatant was discarded and the pellet was resuspended in warm differentiation medium and 10 mL was pipetted into petri dishes. Primary BMDCs were harvested on day 8 by gently pipetting 10 mL of non-adherent cells into a 50 mL Falcon tube. Then 3—10 mL of Accutase (eBioscience) is added to the petri dished for 10 minutes of incubation at 37° and C 5% CO_2 to dislodge the adherent. This was also pipetted into the 50 mL Falcon tube and centrifuged for 10 minutes at 1500rpm and 4°C. The supernatant was discarded and the pellet was resuspended in RPMI culture medium (differentiation medium without GM-CSF), counted with a Neubauer counting chamber and 1 x 10^5 cells were seeded into 96-well plates.

CD4 T-cells were enriched via negative magnetic cell separation and sorted into effector and regulatory CD4 T-cell subsets similar to the irradiation method. Teffs were labelled with CellTrace™ Violet and co-cultured with BMDCs and IL-10-producing TFH, TR1 or Treg cells in 96-well plates. The co-cultures were then stimulated with 40 µg/ml P25 peptide (Bianca Schneider, RCB) to induce proliferation and/or suppression of antigen-specific CD4 T-cells. Flow cytometric measurements were performed 4 days after co-culture and stimulation.

2.2.2.2.9 Gating strategy for FoxP3$^-$ regulatory CD4 T-cells

Unless otherwise described, the scheme in Fig. 2 shows the gating strategy used for the characterisation of FoxP3$^-$ regulatory CD4 T-cells within the scope of this thesis.

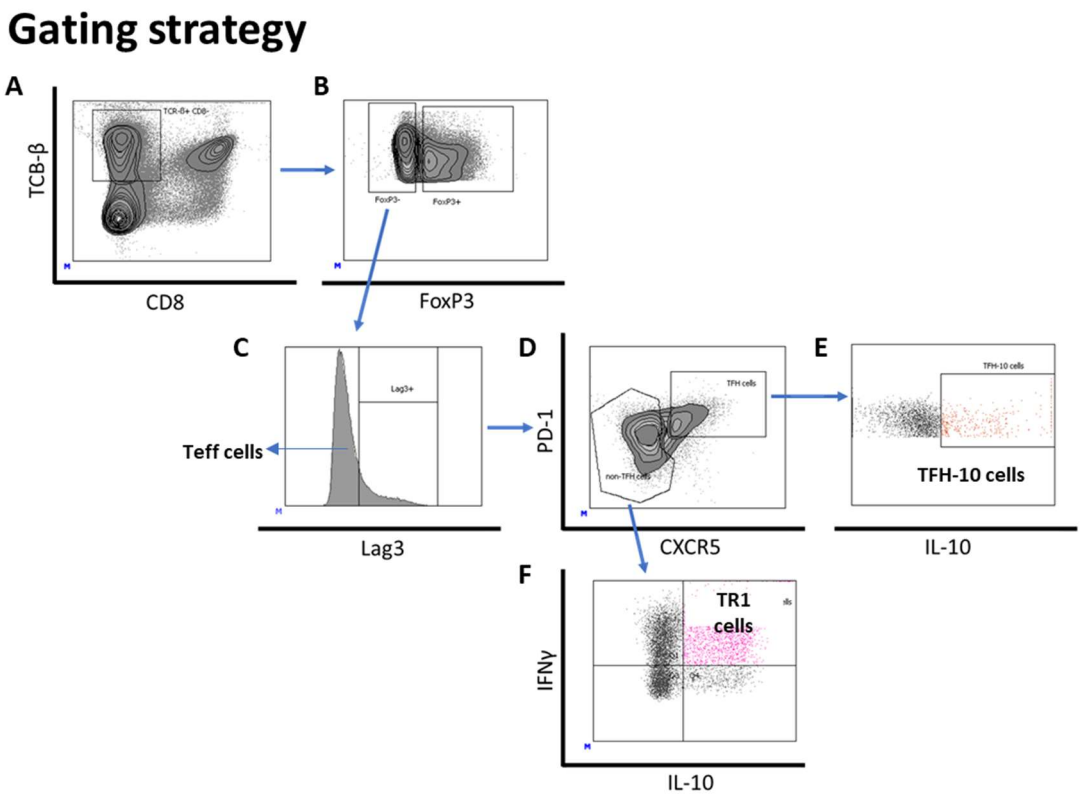

Figure 2. Gating strategy for the characterisation of FoxP3$^-$ regulatory CD4 T-cells
Single cells suspensions from spleen were stimulated for 5 h with PMA/Ionomycin/Brefeldin A block cocktail, stained with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10. f Flow cytometric analysis was performed using LSRII and FlowJo 10.7.0 software. After doublet exclusion (not shown), CD4 T-cells were gated away from TCR-β$^+$ CD8$^-$ (CD4$^+$) population **(A).** From this CD4 T-cell population, FoxP3$^-$ cells **(B)** were gated for LAG3 expression**(C)** and sent the PD-1/CXCR5 plot**(D)**. Cells that were positive for both PD-1 and CXCR5 were further gated on their IL-10 production (TFH-10 cells) **(E)** while cells that were positive for PD-1 but negative for CXCR5 were gated on IFNγ and IL-10 and cells double positive for IFNγ and IL-10 were classed as TR1 cells **(F).** cells negative for LAG3 expression were classified as effector T-cells in proliferation and suppression assays

2.2.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

Blood from vena cava of mice was collected into serum separator tubes (KABE LABORTECHNIK) and centrifuged for 10 minutes at 6000 rpm and 4°C. Concentrations of cytokines and soluble cytokine receptors were detected in serum using standard and multiplex ELISAs

STANDARD ELISA (DuoSet): 96-well microplates (Thermo Scientific Nunc) were coated with the capture antibody and incubated over night at room temperature. The next day, the wells were washed with wash buffer and blocked for 1 hour at room temperature. After blocking, serum samples were incubated in the capture antibody-coated-wells for 2 hours at room

temperature, followed by a 2-hour incubation at room temperature with detection antibody. Subsequently, the samples were incubated with streptavidin-horseradish peroxidase for 20 minutes to enable substrate detection. An ELISA stop solution was added to the mix to stop the reaction and the plate was read immediately with a TECAN reader.

MULTIPLEX ELISA: Mesoscale discovery (MDS) electrochemiluminescence technology was used to measure multiple cytokines simultaneously. The 96 wells of the MSD U-plex plate are equipped with electrodes so that when the complex of secondary antibody, antigen and the capture antibody is formed, the ruthenium ion linked to the secondary antibody is close enough to the electrode to trigger an oxidation-reduction reaction that generates light and can be detected by a camera.

Experiment was performed according to the manufacturer's instructions. First, the linkers were assigned to specific biotinylated capture antibodies vortexed and incubated for 30 minutes. The linkers and biotinylated capture antibodies were then incubated for 30 minutes in 200 µL of MDS stop solution. The wells were then coated with this linker-biotin-capture antibody solution for 1 hour at room temperature and afterwards washed 3 times with ELISA wash buffer.

Calibration standards were prepared according to MDS protocol. Calibration standard and samples were added to separate biotin-capture antibody coated wells and incubated for 1 hour. Thereafter, the 96-plates were washed 3 times with ELISA wash buffer and prepared for detection by further incubating samples in detection antibody at room temperature for 1 hour and afterwards adding the MDS read buffer for immediate measurement on an MDS luminescence reader (MESO QuickPlex SQ 120).

2.2.3 Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0.1 (GraphPad Prism Software, San Diego, USA). Statistical significance was determined by Mann-Whitney Rank Sum Test or analysis of variance (ANOVA) (see figure legends), with a significance set at $P \leq 0.05$.

3 RESULTS

3.1 Increase in regulatory T-cell subsets leads to increased IL-10 production with advancing age

The orchestration of an effective immune response is highly dependent on the tight control of inflammatory processes, as alterations in key regulatory steps could lead to inadequacies in immune response to diseases, infections and even vaccines. A vast body of research has shown that aging is associated with changes to immune responses especially in the T-cell compartment[66,156]. One such change is cytokine dysregulation leading to an increase in regulatory T-cell subsets that supress effector T-cell responses via IL-10 production[90]. Research so far has yet to fully elucidate, which regulatory T-cell subsets are involved in increasing levels of circulating IL-10 with age or the mechanisms involved in the above-mentioned change. Therefore, in the context of this thesis, alterations in cytokine production and thus, regulatory T-cell populations were investigated in aged mice.

3.1.1 Increased levels of IL-10 in aged mice

Cytokines play an important role in balancing immune responses and maintaining immune homeostasis. With aging, a skewing towards a more pro-inflammatory state has been reported but also increases in anti-inflammatory cytokine production. Using young and aged mice, the serum concentration of IL-10 were investigated. In the present thesis, results obtained mimicked what has previously been reported in the literature with older mice showing a significant increase in IL-10 (Fig. 3A). To determine the site of IL-10 production, *IL10* mRNA expression in various lymphoid and non-lymphoid tissues and organs of young and old mice was investigated (Fig. 3B). *IL10* mRNA expression was significantly increased in the lymph nodes, spleen as wells as the epididymal white adipose tissue. This data indicates that systemic levels of IL-10 increase with age and major contributors to this increase are secondary lymphoid organs and epididymal white adipocytes.

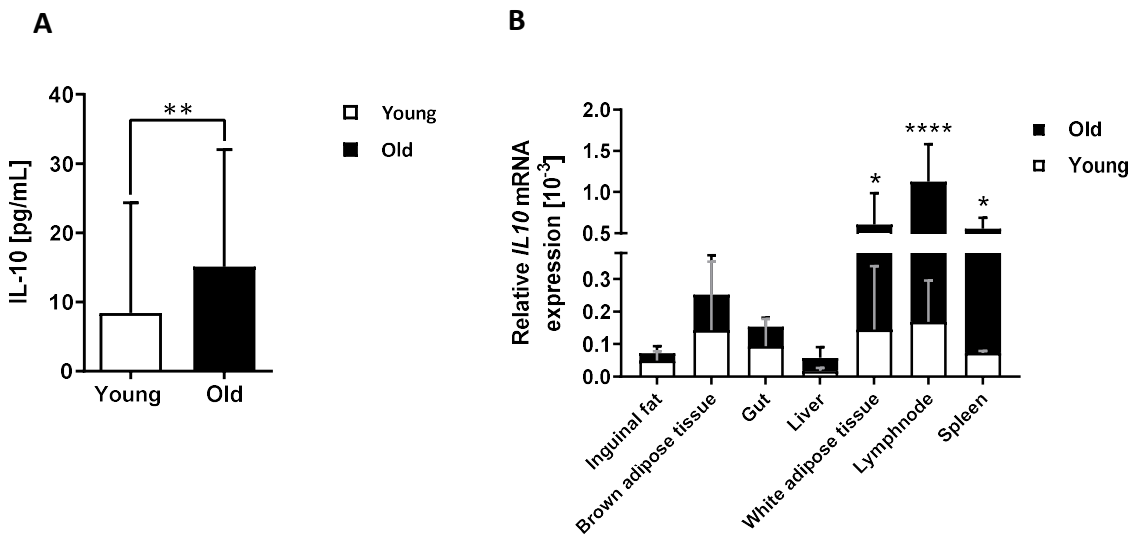

Figure 3. Advancing age results in an increased production of IL-10
(A) Basal IL-10 production in serum samples of young (4 mths old) and old (16 mths old) C57BL/6 mice were measured via ELISA. Mice were euthanised, vena cava blood was collected, and serum separated from the whole blood. **(B)** mRNA from lymphoid and non-lymphoid tissues of young (2 mths old) and old (21 mths old) C57BL/6 mice was isolated, reverse transcribed to cDNA and investigated by qRT-PCR for the relative gene expression of *IL10* in relation to *Rps14 (S14)* as housekeeping gene. Data in bar graph represents the mRNA expression in specific organs or tissues of young and old mice and are shown as mean ± SD of 5-9 mice per group pooled from two independent experiments. (* $P \leq 0.02$, ** $P \leq 0.01$, **** $P \leq 0.0001$; Mann-Whitney Rank Sum Test)

3.1.2 IL-10 production with age stems from T-cells

To ensure that the increased IL-10 production with age was not a result of B-cells, specifically regulatory B-cells (Bregs) interference, old gender and aged matched $CD19^{cre}$ x $IL\text{-}10^{f/f}$ mice that were either Cre-positive (Cre^{+}) or Cre-negative (Cre^{-}) in IL-10 production by B-cells[157] were utilised to quantify IL-10 production in T and B-cell populations using flow cytometry techniques. As seen in Fig. 4A - B, there was no significant difference in IL-10 production by T- or B-cell populations within or between the groups of Cre^{+} and Cre^{-} mice. What was also observed, was that B-cells from both Cre^{+} and Cre^{-} mice produced the least amount of IL-10 in comparison to the other two examined lymphocyte populations. Although no statistical significance was established between the immune cell groups, the result alludes to an increased IL-10 production by CD4 T-cells compared to CD8 T-cells or B-cells with age and reflects what has previously been reported in the literature[121,158].

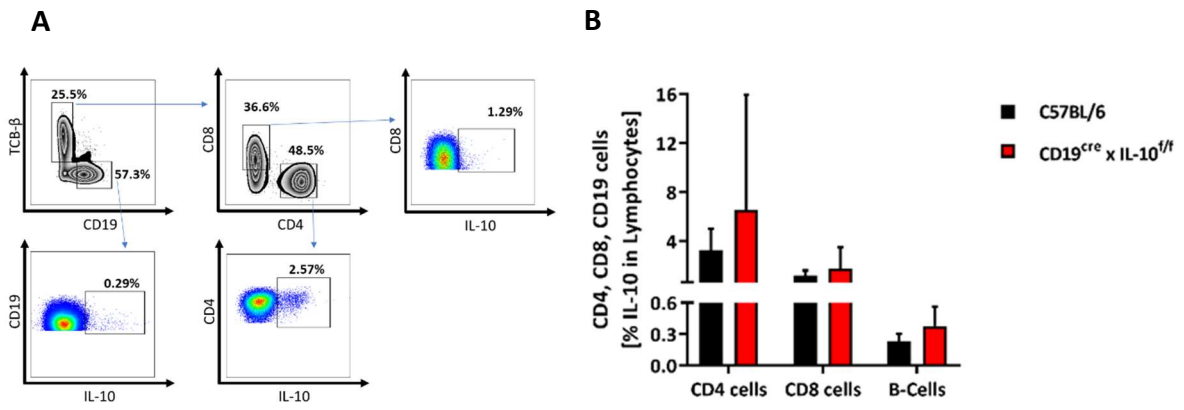

Figure 4. CD4 T-cells account for most IL-10 production in the lymphocyte population
(A) A representative contour plot from the analysed data of a C57BL/6 mouse is shown. **(B)** Spleen cells from old C57BL/6 and $CD19^{cre}$ x $IL\text{-}10^{f/f}$ male mice (15 mths old) were harvested, stimulated for 5h with PMA/Ionomycin and blocked with Brefeldin A. The cells were then fluorescently labelled with Abs against TCR-β, CD8, CD4, CD19 and IL-10 and the percentages of IL-10 within CD4, CD8 and CD19 cells lymphocyte population were analysed by flow cytometry. Data are shown as mean ± SD of 4 mice per group

From the results so far, the lymphocyte population of CD4 T-cells was identified, albeit without statistical significance, as predominant IL-10 producers in aging. For the rest of this study, the focus was therefore placed on the changes that occur with age in CD4 T-cells. To confirm an increase in IL-10 with age, single cell suspensions from spleen cells of young and old WT mice were stimulated and analysed by flow cytometry. After stimulation, the relative frequency (Fig. 5A – B) and number (Fig. 5C) of IL-10-producing CD4 T-cells was determined. IL-10 production was found to be significantly higher in older mice. Given that IL-10 has immune regulatory mechanisms, the source of IL-10 within CD4 T-cells was examined in the conventional $FoxP3^+$ and non-conventional $FoxP3^-$ regulatory populations. While both $FoxP3^+$ and $FoxP3^-$ populations produced IL-10, a substantial amount of IL-10 was detected in the $FoxP3^-$ population of older mice (Fig. 5D, E). Surprisingly, when comparing IL-10 produced by the $FoxP3^+$ population in old mice (Fig. 5D, F), significantly less IL-10 was produced from old $FoxP3^+$ cells than from old $FoxP3^-$ cells (Fig. S1). These results unmask $FoxP3^-$ CD4 T-cells as a potential source for increased IL-10 production during aging.

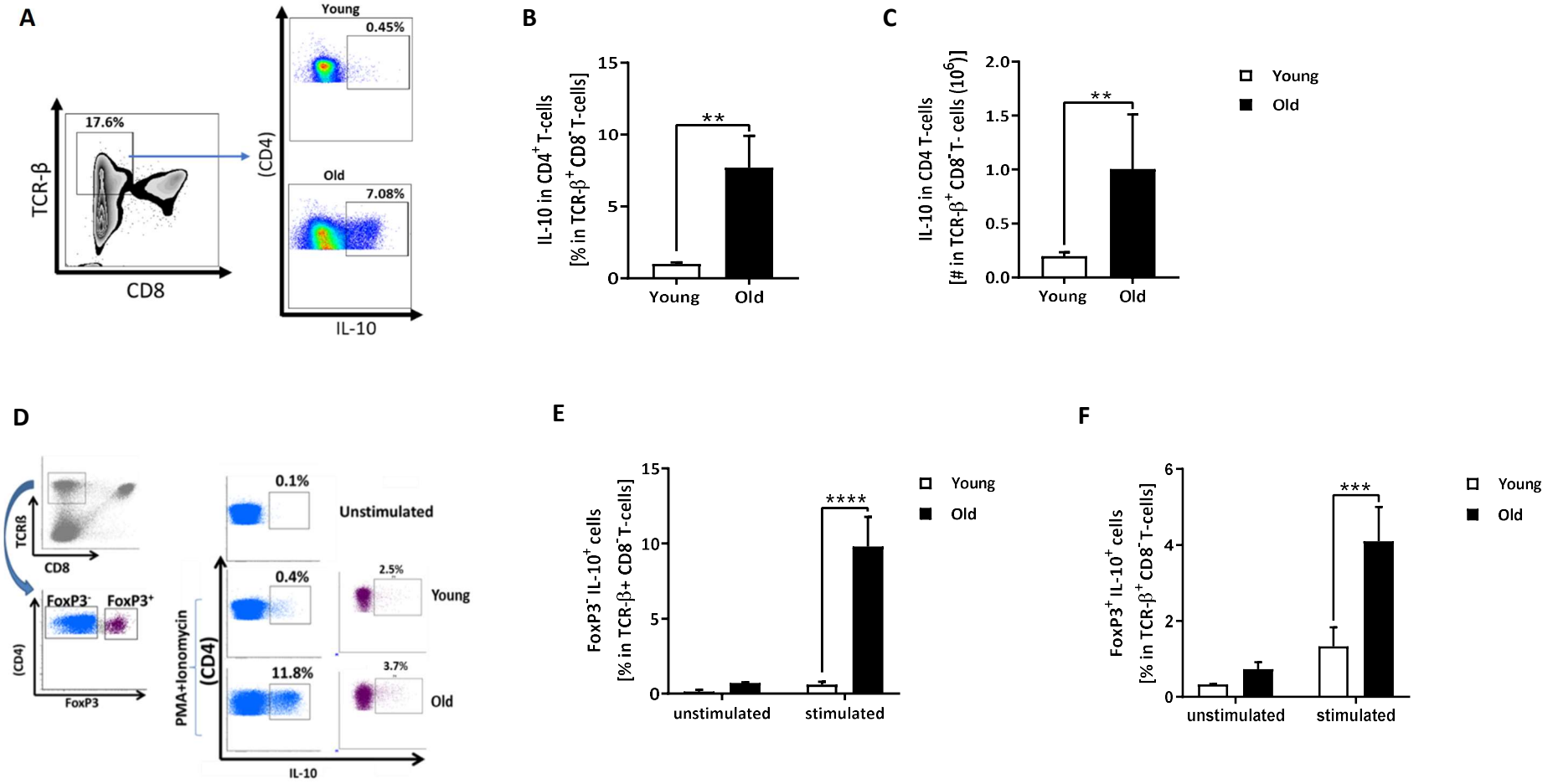

Figure 5. The increase of IL-10 with age can be attributed to its production in FoxP3$^-$ CD4$^+$ T-cells

(A) Representative contour plot of the analysed data. After a 5 h stimulation with PMA/Ionomycin in the presence of Brefeldin A, single cell suspension of spleen cells from young (2 mths old) and old (17 mths old) C57BL/6 mice were fluorescently labelled with Abs against TCR-β, CD8, FoxP3 and IL-10. Using Flow cytometry, the relative percentage of IL-10 **(B)** and the absolute number of IL-10-producing cells **(C)** in the TCR-β$^+$ CD8$^-$ (CD4$^+$) population was determined. **(D)** Representative dot plots of the analysed data used to quantify IL-10 production in FoxP3$^+$ and FoxP3$^-$ populations of CD4$^+$ T-cells. **(E)** The bar graph shows the relative proportions of IL-10 production in TCRβ$^+$ CD8$^-$ FoxP3$^-$ population while **(F)** shows the relative proportion of IL-10 production in TCRβ$^+$ CD8$^-$ FoxP3$^+$ population. Results are shown as mean ± SD of 3 - 8 mice per group and represents 1 of 4 experiments. (** P ≤ 0.04; Mann-Whitney Rank Sum Test; *** P ≤ 0.001, **** P ≤ 0.0001; Two-way ANOVA with Sidak's multiple comparison test)

3.1.3 Characterisation of FoxP3$^-$ IL-10$^+$ CD4 T-cells in young and old mice

Based on cells described in the literature, 4 potential origins or populations of the IL-10-producing FoxP3$^-$ CD4 T-cells were envisioned. Namely, exTH17-cells and exTreg cells due to their high instability and plasticity, TR1 T-cells and TFH as both cell types have the tendency to produce the anti-inflammatory cytokine IL-10[90,148,159,160]. To track the fate of TH17 and FoxP3$^+$ CD4 T-cells towards FoxP3$^-$ regulatory cell conversion *in vivo*, young and old fate mapping *Rosa26R*^{YFP} x *IL-17A*^{Cre} (exTH17) and *Rosa26R*^{dTomato} x *FoxP3*^{Cre} (exTregs) mice were used to track TH17 and Treg cells that lose their initial function to become FoxP3$^-$ IL-10 producers. In exTH17 mice, the frequencies of TH17-cells that converted to FoxP3$^-$ IL-10 producers were similar in both age groups with less than 0.1% of the TH17-cells being converted (Fig. 6A). In exTreg mice, about 14.6% of FoxP3$^+$ cells lost the expression of this transcription factor to become FoxP3$^-$ IL-10-producing cells, but like the exTH17-cells, there was no significant difference between young and old mice when FoxP3 was lost (Fig. 6B). Because the percentages of cells that lost either their TH17 or FoxP3 markers to become FoxP3$^-$ IL-10-producing CD4 T-cells did not differ between young and old, these results indicate that a switch in cell functionality does not account for increased IL-10 production in FoxP3$^-$ CD4 T-cells cells with age.

Studies have shown that a subpopulation of FoxP3$^-$ IL-10$^+$ CD4 T-cells are produced in an IL-21- dependent manner and although many subpopulations of CD4 T-cells produce IL-21 the highest production of this cytokine is found in the TFH population[161,162]. Roncarolo et al., one of the first to report on TR1 cells, gave indication to these cells as prominent IL-10 producers[93]. Consequently, TFH and TR1 cell frequencies in the spleen of young and old mice were investigated in the present thesis. It was observed that older mice had significantly higher amounts of TR1 as well as TFH cells (Fig. 6C), suggesting, TR1 and TFH cells to be the source of increased IL-10 in the FoxP3$^-$ CD4 T-cell compartment. From this point on, TFH cells positive for IL-10 will be referred to as **TFH-10** cells.

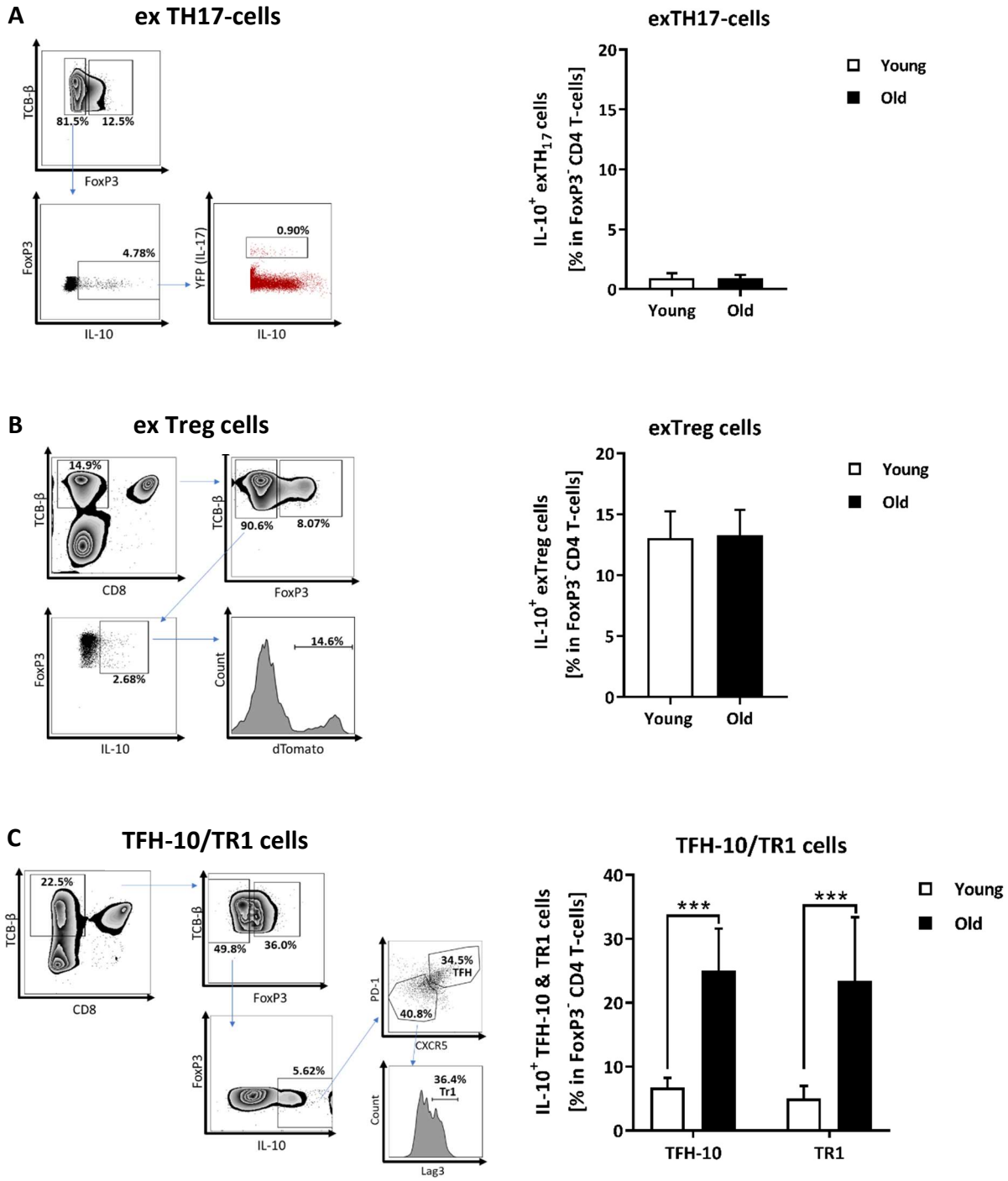

Figure 6. TFH-10 and TR1 cells but not exTregs or exTH17-cells account for the increased IL-10 production in FoxP3$^-$ CD4 T-cells in older mice

After a 5 h incubation with PMA/Ionomycin and Brefeldin, single cell suspension of spleen cells from young (6 – 12 wks old) and old (12 mths old) *Rosa26R*^{YFP} x *IL-17A*^{Cre}, *Rosa26R*^{dTomato} x *FoxP3*^{Cre} and C57BL/6 mice were fluorescently labelled with Abs against TCR-β, CD8, FoxP3, IL-17 and IL-10 for exTH17 **(A)**, TCR-β, CD8, FoxP3, and IL-10 for exTregs **(B)**, TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10 for TFH-10/TR1 cells **(C).** Subsequently, flow cytometric analysis was performed. The plots and graphs show the relative frequencies of the indicated subsets of FoxP3$^-$ IL-10$^+$ within TCR-β$^+$ CD8$^-$ (CD4$^+$) T-cells. The results depicted above represents 1 of 2 - 3 independent experiments. Results are shown as mean ± SD of 4 - 5 mice per group. (*** P ≤ 0.001; Two-way ANOVA with Sidak's multiple comparison)

Aging reshapes cytokine profiles in mice, with increased IL-10 production by CD4 T-cells being a typical feature. However, this IL-10 does not originate from conventional FoxP3$^+$ regulatory CD4 cells, but mainly from the population of FoxP3$^-$ regulatory CD4 T-cells described in the present thesis. Indeed, the experimental design in the present thesis determine TFH-10 and TR1 cells as the source of increased IL-10 during aging while categorically excluding TH17 and Treg cell as possible sources for increased IL-10 levels in older mice.

In a previous study, Jun-O Jin et al. showed a relationship between IL-6 and IL-10 production by FoxP3$^-$ regulatory CD4 T-cells *in vitro*[95]. Because systemic levels of IL-6 usually increases with age, increased IL-10 production by FoxP3$^-$ CD4 T-cells in older mice suggest a role of IL-6 in the accrual or maintenance of this CD4 T-cells subset.

3.2 The role of IL-6 and its signalling pathways in the accrual of FoxP3$^-$ IL-10$^+$ CD4 T-cell

The association between aging and a lot of age-related diseases coincides with inflammation. During aging, there is a steady increase in the level of inflammatory mediators such as TNF, CRP and most notably, IL-6[105]. To test if the increased FoxP3$^-$ IL-10$^+$ CD4 T-cell populating found in older mice is as a result of increased IL-6 as observed in the in vitro study by Jun-O Jin et al.[95], in the present thesis, the direct influence of a lack in IL-6 on FoxP3$^-$ IL-10$^+$ CD4 T-cells was investigated. Effort was put into understanding the IL-6 signalling cascade and the role its different signalling pathways play in the accrual of FoxP3$^-$ regulatory cells with age. Because TFH-10 and TR1 cells induced by IL-21 and IL-27 respectively[93,148], the role of IL-6 in the production of both cytokines in the context of aging was also investigated.

3.2.1 IL-6 increases with advancing age

Increases in the circulating levels of certain cytokines such as IL-6, CRP, TNF is directly linked to the aging phenotype of immune systems[105]. To find out if IL-6 is altered with age, the present thesis compares IL-6 production in young and old C57BL/6 mice using ELISA and qRT-PCR. Results obtained from the analysis of serum samples showed a propensity for increased IL-6 production with age (Fig. 7A). Tissue localisation of the increased IL-6 production was determined by qRT-PCR. Similar to the elevated *IL10* mRNA expression (see Fig. 3B), *IL6* mRNA expression, although not statistically significant, was also increased in white, brown adipose tissue and spleen in older mice compared to their younger counterparts.

However, in the lymph nodes of old mice, a significant increase in *IL6* mRNA expression was observed (Fig. 7B).

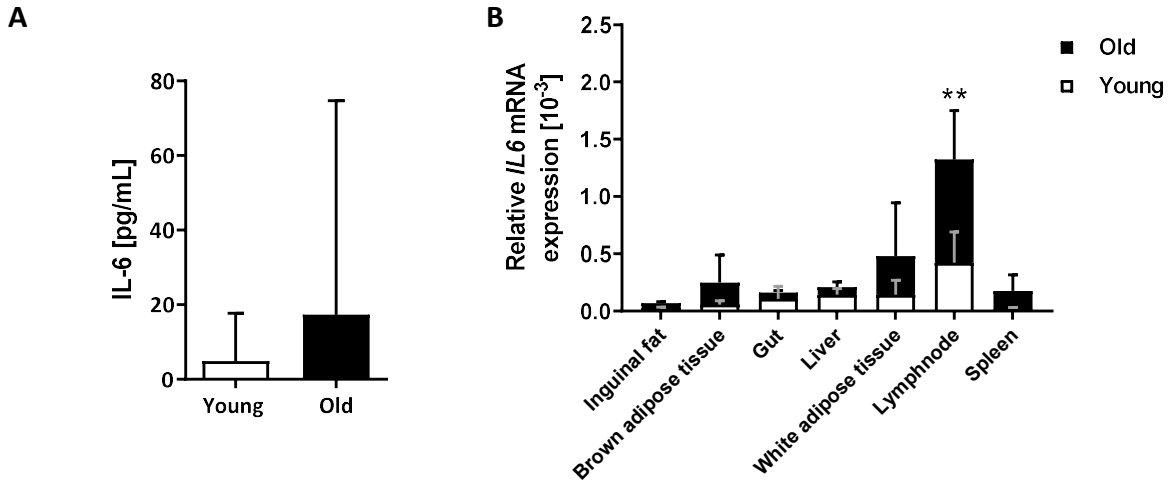

Figure 7. IL-6 secretion increases in old mice
(A) Basal IL-6 production in serum samples of young (4 mths old) and old (16 mths old) C57BL/6 mice were measured via ELISA. Mice were euthanised, vena cava blood was collected, and serum separated from the whole blood. Results are shown as mean ± SD of 5-9 mice per group pooled from two independent experiments. **(B)** RNA from lymphoid and non-lymphoid tissues of young (2 mths old) and old (21 mths old) C57BL/6 mice was isolated, reverse transcribed to cDNA and investigated by qRT-PCR for gene expression of *IL6* in relation to *Rps14* as the housekeeping gene. Result of the relative mRNA expression in the specific organs or tissues in young and old mice and are shown as mean ± SD of 5-9 animals per group pooled from two independent experiments. (** P <0.01; Mann-Whitney Rank Sum Test)

3.2.2 Loss of IL-6 impacts the accrual of FoxP3$^-$ IL-10$^+$ CD4 T-cells with age

In the present study, it was demonstrated that within the CD4 T-cell subset, FoxP3$^-$ cells account for most of the IL-10 production with age (see Fig.5D), and a body of work has shown that the "gerontologist" cytokine IL-6 increases with age and is associated with elevated IL-10 production[123,163]. To eventually prove that the accrual of IL-10-producing FoxP3$^-$ CD4 T-cells is IL-6-dependent, young and old C57BL/6 and IL-6 deficient (IL-6$^{-/-}$) mice were compared.

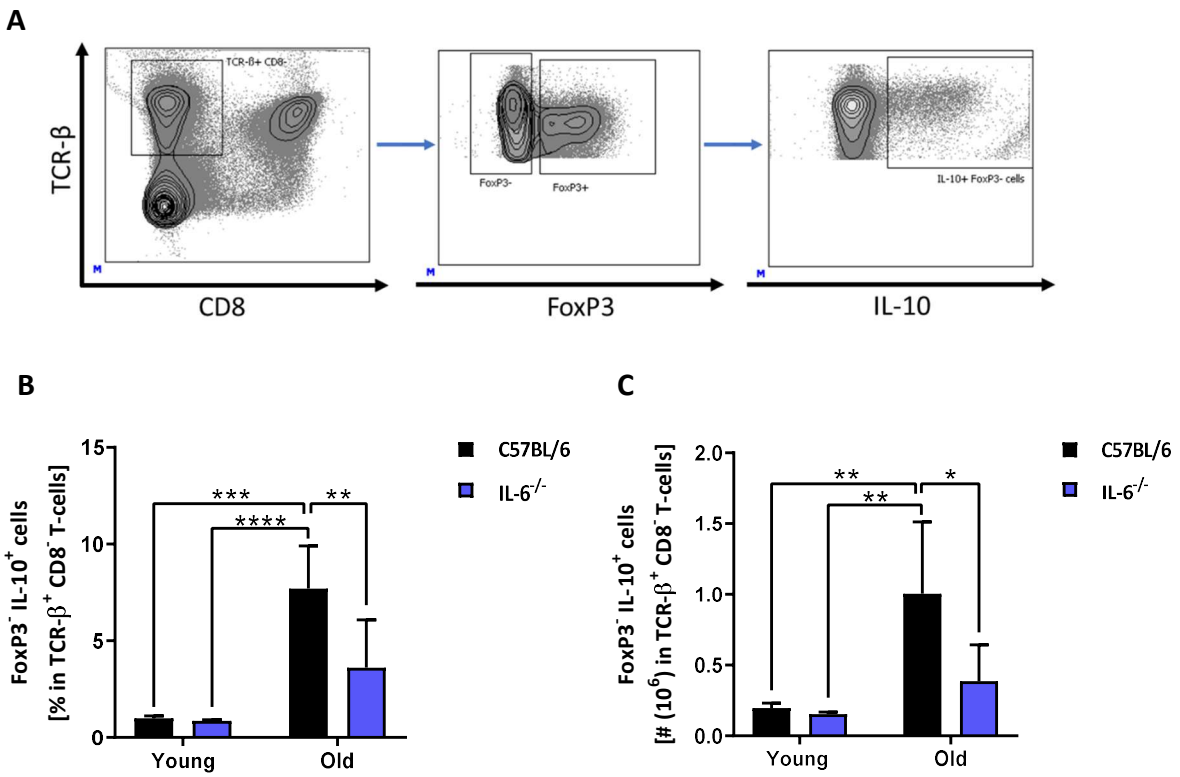

Figure 8. The frequency of IL-10 production in FoxP3$^-$ CD4 T-cells decreases in older mice with the loss of IL-6.

Representative contour plot of the analysed data **(A)**. After a 5 h stimulation with PMA/Ionomycin and blocking with Brefeldin A, single cell suspension of spleen cells from young (2 mths old) and old (17 mths old) C57BL/6 and IL-6$^{-/-}$ mice were fluorescently labelled with Abs against TCR-β, CD8, FoxP3 and IL-10. The relative percentage of IL-10 **(B)** and the absolute number of IL-10-producing cells in the FoxP3$^-$ CD4 T-cell population **(C)** was determined by flow cytometry. Results depicted above represents 1 of 2 independent experiments. Data are shown as mean ± SD of 4 – 8 mice per group. (* P ≤ 0.01, ** P ≤ 0.004, *** P ≤ 0.0001, **** P ≤ 0.001; Two-way ANOVA with Tukey's multiple comparison)

While no significant difference was observed in the frequency or number of FoxP3$^-$ IL-10$^+$ CD4 T-cells between young mice, old IL-6$^{-/-}$ mice had a marked reduction in IL-10 production by FoxP3$^-$ CD4 T-cell population both in frequency and number when compared to their WT C57BL/6 wildtype mice (Fig. 8B - C). Overall, this data shows that IL-6 is indeed important for the accrual of IL-10-producing FoxP3$^-$ CD4 T-cells with age.

3.2.3 Classical stimulation of STAT1 and STAT3 by murine IL-6 is decreased in older mice

Amongst the numerous signalling pathways activated by IL-6, the most prominent pathway is the JAK2/STAT. Under the STAT proteins, IL-6 is mostly associated with STAT1 and STAT3 activation[128]. Therefore, in the present thesis, it was predicted that the increase in FoxP3$^-$ IL-10$^+$ CD4 T-cells (TFH-10 and TR1 cells) would be ascribed to elevated IL-6 levels

activating STAT pathways. To investigate this hypothesis, the effect of IL-6 on activation of STAT1 and STAT3 proteins was explored by flow cytometry and ELISA.

After a 30-minute stimulation of single spleen cell suspension with murine IL-6, it was observed that the induction of intracellular levels of tyrosine phosphorylation of STAT1 at position 701 (Fig. 9A) and STAT3 at position 705 in CD4 T-cells (Fig. 9B) was significantly elevated in younger mice. Based on determining the MFI a more enhanced phosphorylation of STAT1 in comparison to STAT3 was observed in CD4 T-cells of younger mice.

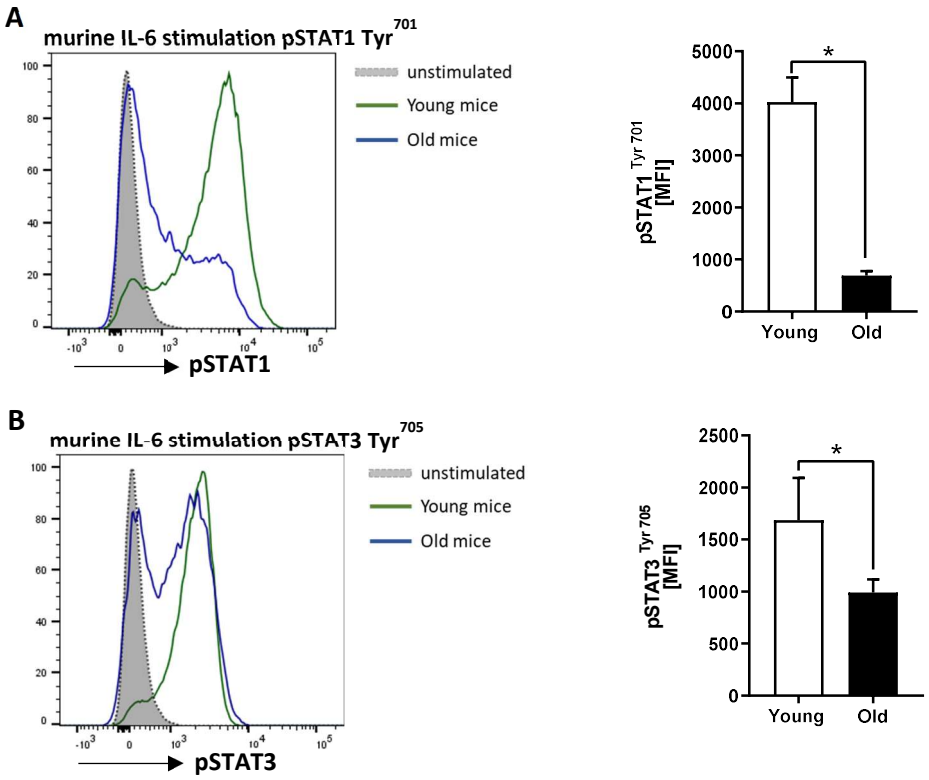

Figure 9. Intracellular pSTAT 1 and pSTAT 3 levels increases in the CD4⁺ T-cell population of younger mice after IL-6 stimulation

After a 30 minute stimulation with murine IL-6, single spleen cell suspension from young (6 wks old) and old (17 mths old) C57BL/6 mice were flourescently labelled with Abs against TCR-β, CD4, pSTAT1 (Tyr701) or pSTAT3 (Tyr705). The MFI of the phpsphorylated STAT proteins were measured and analysed by flow cytometry. The representative histograms shows intracellular pSTAT1 tyr^{701} **(A)** or pSTAT3 tyr^{705} **(B)** level in the unstimulated control (grey), in young (green) and in old (blue) mice while the bar charts depict the MFI of the analysed pSTAT proteins in the CD4 population **(A, B)**. Results are shown as mean ± SD of 4 mice per group and represents 1 of 3 experiments. (* P ≤ 0.03; Mann-Whitney Rank Sum Test)

Because stimulating the cells with murine IL-6 forces the activation of the classical transduction pathway which utilises membrane-bound IL-6Rα and gp130, the expression of both subunits was measured on CD4 T-cells isolated from spleens of young and old mice by flow cytometry. Young mice expressed significantly higher amounts of membrane-bound IL-6Rα in comparison to older mice (Fig. 10A, B). Although no significant difference between

young and old mice was detected, there was a tendency in younger mice to also express more of gp130 on CD4 T-cells (Fig. 10A, C). Collectively, these results suggest a more pronounced role for IL-6 classical signalling in young years, whereas in advanced age, IL-6 appears to signal through an alternative pathway.

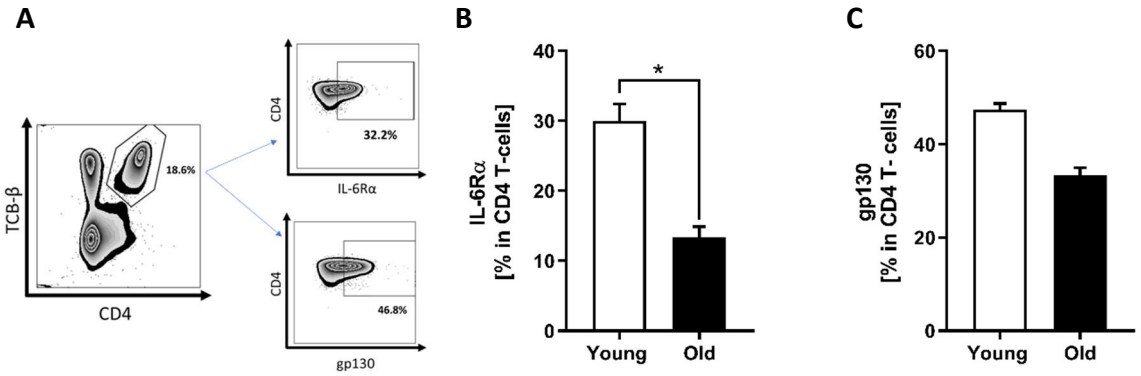

Figure 10. The frequency of IL-6Rα is increased in younger mice
(A) Representative contour plot analysed in young mouse. Single spleen from young (3 mths old) and old (15 mths old) C57BL/6 mice were fluorescently labelled with Abs against TCR-β, CD4, CD126 (IL-6Rα) or gp130. The relative percentage of IL-6Rα **(B)** and gp130 **(C)** expressed on CD4 T-cells was determined by flow cytometry. Results are shown as mean ± SD of 4 mice per group and represents 1 of 3 experiments. (* P <0.03; Mann-Whitney Rank Sum Test).

3.2.4 Transient IL-6 receptor knockdown in IL-6Rα$^{f/f}$ CD4 CreER^{T2} mice does not affect TFH-10 or TR1 populations

To ascertain that the accrual of TFH-10 and TR1 cells in old age is not a result of IL-6 classical signalling, a transient knockdown was performed in transgenic mice expressing a tamoxifen inducible Cre recombinase in CD4 T-cells and a loxp-flanked IL-6Rα gene enabling for the deletion of membrane-bound IL-6Rα when tamoxifen is administered. In the present thesis, 2 mg tamoxifen was administered i.p. for 4 consecutive days to Cre$^+$ or Cre$^-$ old mice. A week after the first injection, mice were sacrificed and flow cytometric analysis was performed (Fig. 11A). Single spleen cell suspension from were prepared and stained with appropriate antibodies. To verify the efficiency of the knockdown after tamoxifen administration, IL-6Rα on CD4 T-cells was measured and a marked reduction in the frequency of IL-6Rα in Cre$^+$ mice compared to Cre$^-$ mice detected (Fig. 11B, C). This result indicates an efficient CD4-specific IL-6Rα knockdown.

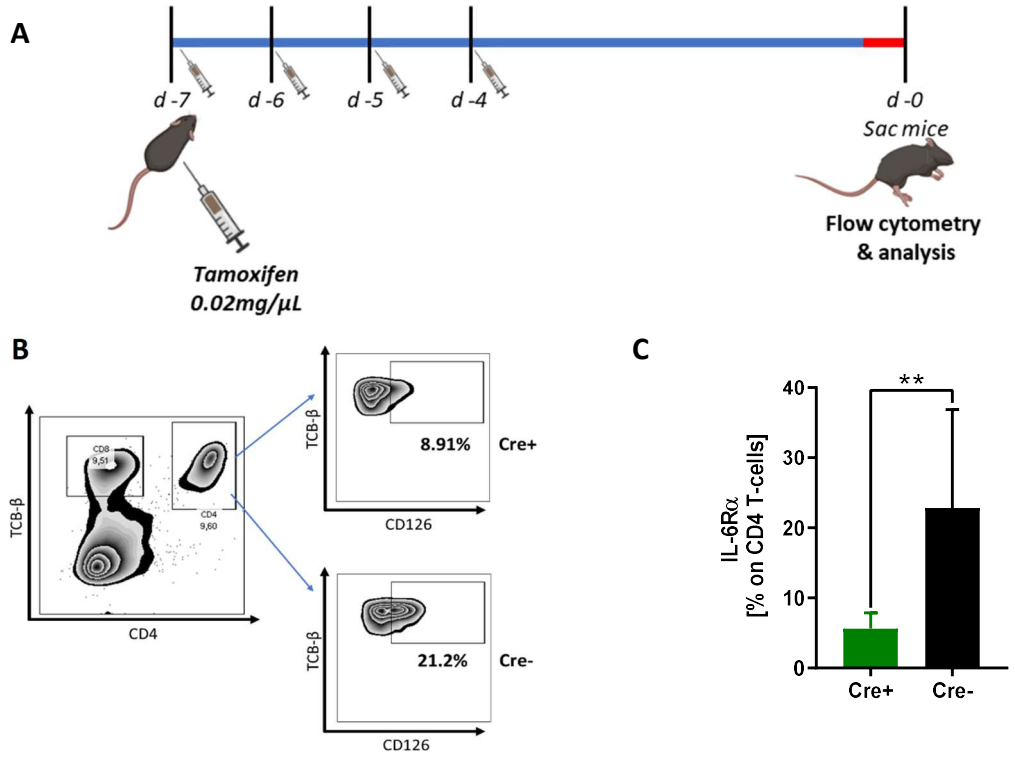

Figure 11. CD4 T-cells specific knockdown of the membrane-bound IL-6Rα
Old IL-6R$^{f/f}$ CD4 creER^{T2+} and IL-6R$^{f/f}$ CD4 creER^{T2-} mice were i.p. treated with tamoxifen or 4 consecutive days. Single spleen cell suspension was obtained 7 days after the first treatment **(A)**. Cells were fluorescently labelled with Abs against TCR-β, CD4 and CD126 (IL-6Rα) and analysed by flow cytometry. Representative plots of the analysed data are shown in **(B)**. The relative proportion of IL-6R within the CD4$^+$ population is presented in the bar graph **(C)**. Results are shown as mean ± SD of 2 - 5 mice per group and represents 1 of 2 experiments (** P ≤ 0.001; Mann-Whitney Rank Sum Test).

After validating the knockdown of the IL-6Rα, the relative proportions of TFH-10 and TR1 cells withing the CD4 T-cell population in old Cre$^+$ and Cre$^-$ mice was then investigated in single cell suspension prepared from spleen tissue and stained for flow cytometry. The frequencies of TFH-10 and TR1 cells in both mice groups were similar (Fig. 12A—C). Although there was no measurable significant difference between Cre$^+$ and Cre$^-$ mice, there was a slight tendency for IL-6Rα competent mice to have higher frequencies of both TFH-10 and TR1 cells. These results further prove that IL-6 classical signalling with STAT phosphorylation on its tyrosine residue is not responsible for the accrual of FoxP3$^-$ IL-10$^+$ TFH-10 and TR1 cells with advancing age.

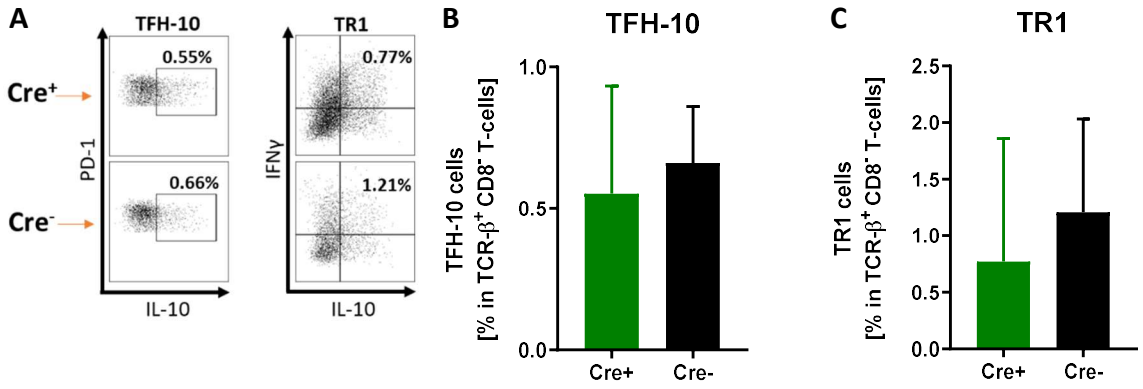

Figure 12. Short term knockdown of IL-6Rα has no effect on the frequencies of TFH-10 or TR1 cell populations in old mice

Single spleen cell suspension from old (17 mths old) IL-6R$^{f/f}$CD4CreER^{T2+} and IL-6R$^{f/f}$CD4CreER^{T2-} mice were stimulated for 5 h with PMA/Ionomycin, blocked with Brefeldin A and fluorescently labelled for flow cytometry with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10. The relative frequencies of TFH-10 (see. Fig 2E) and TR1 (see. Fig 2F) cells in Cre$^+$ or Cre$^-$ mice are shown in the plots and graphs above (A—C). Results are shown as mean ± SD of 2 - 5 mice per group and represents 1 of 2 experiments

Taken together, the IL-6-dependent increase in TFH-10 and TR1 cells with age appears to be mediated by an alternative route or through activation of STAT by inducing phosphorylation on a different amino acid residue.

3.2.5 Increased sIL-6Rα suggests a more active IL-6 trans-signalling pathway in older mice

So far, analysis of STAT phosphorylation and expression of membrane-bound IL-6Rα in the present thesis has shown that the classical IL-6 signalling pathway plays a more prominent role at younger ages. However, IL-6 is also known to signal via the trans-signalling route which drives many inflammatory disorders[164]. Therefore, in the present thesis, sIL-6Rα, which serves as a marker for IL-6 trans-signalling, was measured in serum samples from young and old mice (Fig. 13). Unlike the distribution of membrane-bound IL-6Rα, older mice had significantly higher concentrations of sIL-6Rα circulating in their serum in comparison to younger mice. This increase in serum sIL-6Rα suggests that the IL-6 trans-signalling pathway is increasingly utilised during aging.

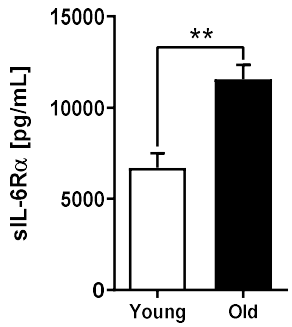

Figure 13. Increased amounts of sIL-6Rα in aged mice

Serum samples prepared from vena cava blood of young (3 mths old) and old (15 mths old) C57BL/6 mice were used to determine the concentration of circulating soluble IL-6Rα by ELISA. Results are shown as mean ± SD of 4 mice per group and are representative of 1 of 2 experiments (** P ≤ 0.01; Mann-Whitney Rank Sum Test).

To prove the hypothesis that for efficient STAT1 and STAT3 protein activation in older mice IL-6 has to signal in trans, lymphocytes from spleen cells of young and old mice were forced to signal through the trans-signalling route. To activate IL-6 trans-signalling, single spleen cell suspensions from young and old mice were stimulated for 30 minutes with HyIL-6. Cells were then stained with antibodies against phosphorylated STAT1 and STAT3 in CD4 population and analysed by flow cytometry. Contrary to the elevated amounts of sIL-6Rα, neither STAT1 nor STAT3 phosphorylation increased in older mice under this stimulatory condition. Indeed, there was no difference in phosphorylation when comparing young and old mice (Fig. 14A, B). Taken together, increased levels of sIL-6 did not lead to increased IL-6 trans-signalling in older mice.

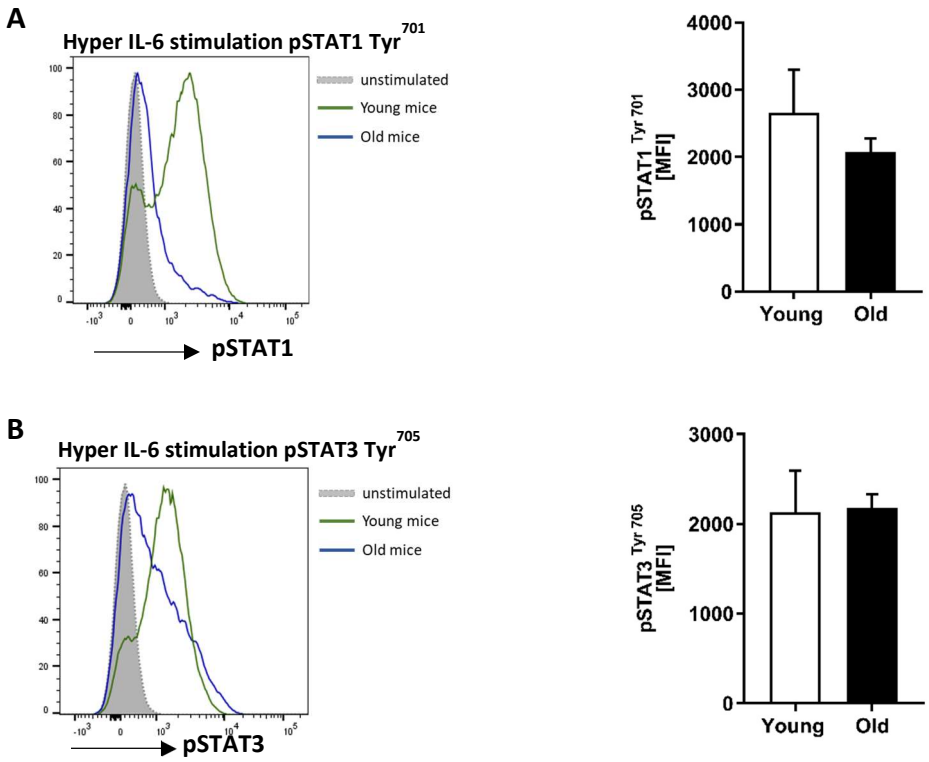

Figure 14. Intracellular pSTAT1 and pSTAT3 levels are similar in CD4 T-cell population of young and old mice after HyIL-6 stimulation
After a 30 minute stimulation with HyIL-6, single spleen cell suspensions from young (6 wks old) and old (17 mths old) C57BL/6 mice were flourescently labelled with Abs against TCR-β, CD4, pSTAT1 (Tyr701) or pSTAT3 (Tyr705).The MFI of the phpsphorylated STAT proteins were measured and analysed by flow cytometry The representative histograms shows intracellular pSTAT1 **(A)** or pSTAT3 **(B)** levels in unstimulated cells (grey) and in stimulated young (green) and old (blue) mice while the bar charts show the MFI of the analysed pSTAT proteins in the CD4 population. Results are shown as mean ± SD of 4 mice per group.

3.2.6 sgp130, the endogenous block for IL-6 trans-signalling is increased in older mice

The naturally occurring trans-signalling inhibitor sgp130 is usually found as a triad complexed with IL-6 and sIL-6R[165]. In the present thesis, it was of interest to know if the lack of increased

STAT1 and STAT3 phosphorylation after HyIL-6 stimulation could be due to inhibition of the trans-signal transduction pathway by sgp130. To this end, serum concentration of sgp130 in young and old C57BL/6 mice from vena cava blood was analysed. Older mice had a 3-fold increase of circulating sgp130 in comparison to their younger counterparts (Fig. 15) suggesting a dampening of IL-6 trans-signalling by sgp130 in older mice.

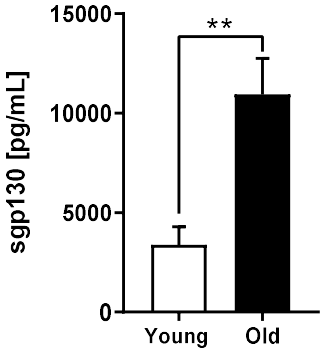

Figure 15. Higher amounts of sgp130 in aged mice
Serum samples prepared from vena cava blood of young (3 mths old) and old (15 mths old) C57BL/6 mice were used to determine the concentration of circulating gp130 by ELISA. Results are shown as mean ± SD of 4 mice per group and is representative for 2 experiments (** $P \leq 0.01$; Mann-Whitney Rank Sum Test).

3.2.7 TFH-10 and TR1 populations are unaltered in the absence of IL-6 trans-signalling pathway

The present thesis revealed so far, that the trans-signalling marker —sIL-6R—and the inhibitor to this pathway—sgp130—increase with age. To re-examine if the accrual of TFH-10 and TR1 cells is mediated by IL-6 trans-signalling, percentages of both cell populations were analysed in aged mice with a constitutive block in the trans-signalling pathway (PEPCU-sgp130) and compared to wildtype mice. To this end, single spleen cell suspensions were prepared, incubated in media, or stimulated with PMA/Ionomycin and stained with antibodies for flow cytometric analysis. The relative proportion of TFH-10 was found to be comparable in both PEPCU-sgp130 and wildtype mice (Fig. 16A-B). A similar phenomenon was observed when TR1 cells were examined (Fig. 16C).

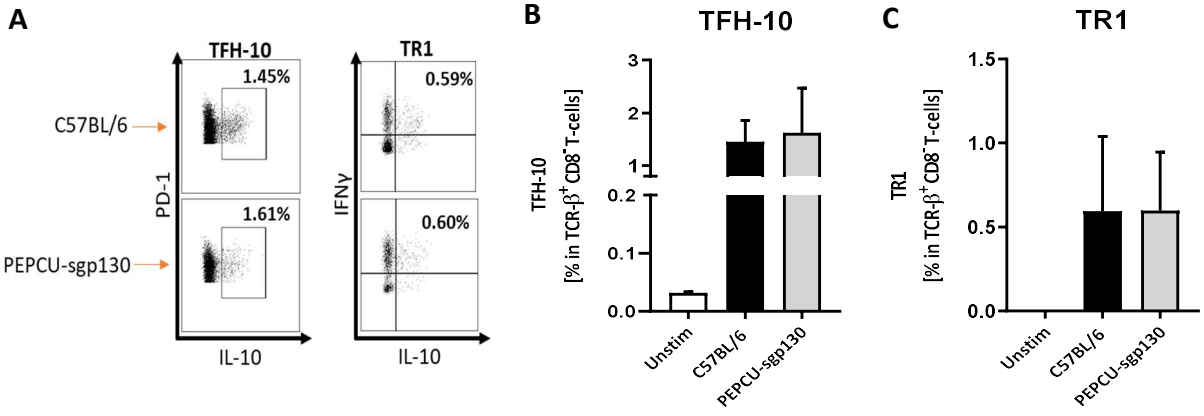

Figure 16. The frequency of TFH-10 and TR1 cells is unchanged between aged WT and trans-signalling deficient mice

Single spleen cell suspensions of C57BL/6 (18 mths old) and PEPCU-sgp130 (18 mths old) mice were stimulated for 5 h with PMA/Ionomycin, blocked with Brefeldin A and stained for flow cytometric analysis with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10. The relative proportions of TFH-10 **(A and B)** and TR1 **(A and C)** in the TCR-β^+ $CD8^-$ ($CD4^+$) population are shown in the plots and bar graphs above (for TFH-10 and TR1 gating, see Fig. 2E and 2F). Results are shown as mean ± SD of 3 – 6 mice per group.

3.2.8 Classical stimulation of STAT 3^{Ser727} by IL-6 is increased in older mice

STAT3, through the activity of various kinases, is also sensitive to phosphorylation on its serine residue at position 727 (Ser727). One such kinase that is able to phosphorylate STAT3 on its serin residue is the mammalian target of rapamycin (mTOR)[166]. mTOR forms a complex with mTORC1 and mTORC2. While mTORC1 activation promotes cell proliferation leading to replicative aging[167,168], mTORC2 activity promotes an age-related decline in CD4 T-cell function[169]. To test a possible effect of IL-6 on CD4 T-cell after STAT3 phosphorylation at the Ser727 residue, single spleen cell suspensions of young and old mice were prepared, stimulated for 30 minutes with IL-6 and stained with abs for flow cytometric analysis. The proportion of cells within the CD4 T-cell population that was positive for phosphorylated STAT3 Ser727 was determined (Fig. 17A, B). Although no statistical significance was found, the results showed increased phosphorylation of Ser727 in older mice compared to younger mice, which contrasts with IL-6-mediated phosphorylation of STAT3 Tyr705, inferring that in older mice the effect of IL-6 on STAT3 is more pronounced at the serin residue.

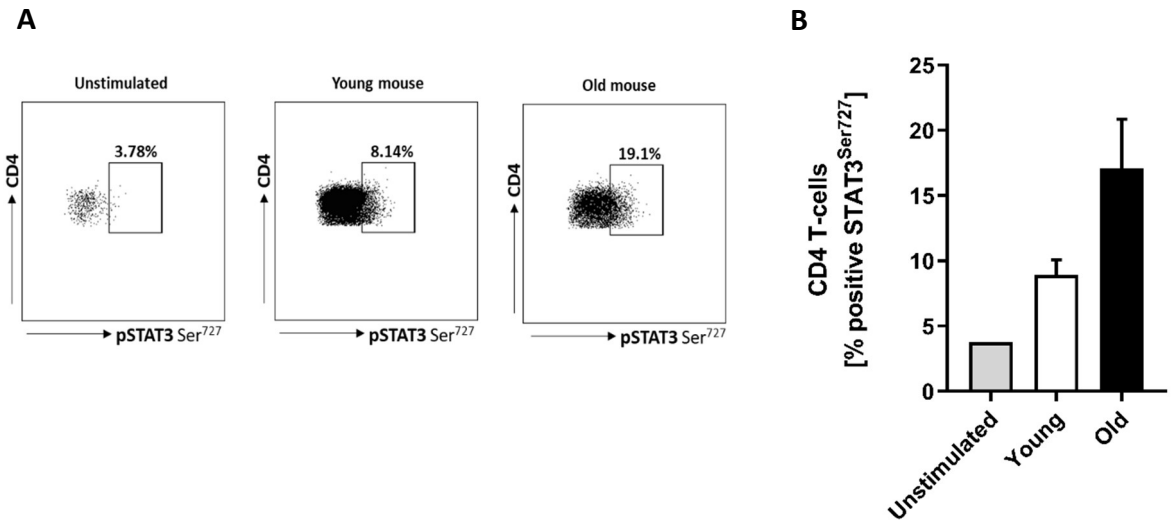

Figure 17. After IL-6 stimulation, intracellular pSTAT 3^{Ser727} levels increase in CD4 T-cells of older mice
Single cell suspension of spleen cells from C57BL/6 young (5 mths old) and old (13 mths old) mice were stimulated for 30 minutes with IL-6 and stained for flow cytometric analysis with Abs against TCR-β, CD4 and pSTAT3 (Ser727). The representative dot plots **(A)** and bar chart **(B)** show the percent of positive intracellular pSTAT3 CD4 T-cells in the unstimulated cells (grey bar) and pSTAT-3 in stimulated young (white bar) and old (black bar) mice. Data are shown as mean ± SD of 2- 4 mice per group

3.2.9 Transient STAT3 KD in STAT3$^{f/f}$ CD4 CreER^{T2} mice does not influence TFH-10 or TR1 cell frequencies

So far, the present thesis revealed that more STAT3 is activated in young mice when classical IL-6 signalling leads to phosphorylation on the tyrosine residue. In contrast, older mice show a tendency for increased STAT3 activation when the serin residue is phosphorylated. Prompted by these differences, the effect of a STAT3 knockdown on the frequencies of TFH-10 and TR1 cells in old mice was investigated. Because constitutive STAT3-deficient mice are not viable[170], a transient STAT3 knockdown using transgenic mice expressing a tamoxifen-inducible Cre recombinase (CreER^{T2}) were used. Cre^{+} as well as control Cre^{-} mice were injected i.p for 5 successive days with 2mg tamoxifen and sacrificed one week after the first injection was administered. Immediately after the mice were euthanised, single spleen cell suspensions were prepared, fluorescently labelled for flow cytometry and analysed (Fig. 18A). The amount of STAT3 produced per cell was reduced in the Cre^{+} mice (Fig. 18B, C), and thus, validating the success of the induced transient knockdown of STAT3.

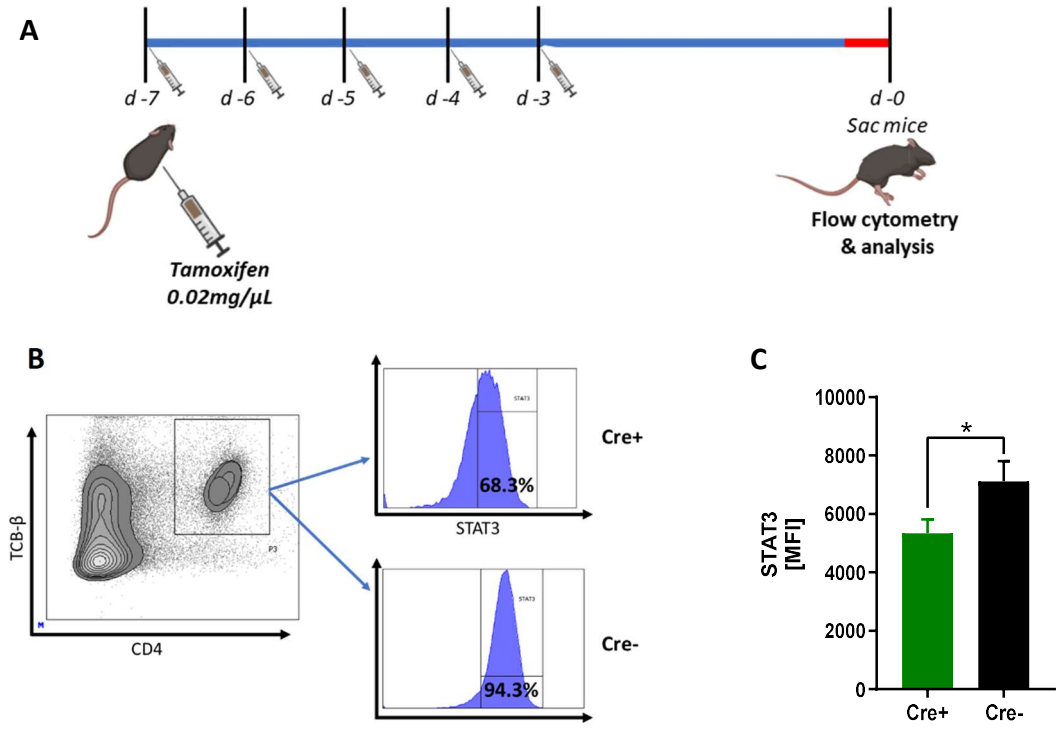

Figure 18. Reduced STAT3 expression in STAT3$^{f/f}$ CD4 creER^{T2} mice after 5 days of tamoxifen treatment
Old STAT3$^{f/f}$ CD4 creER^{T2} mice were treated with tamoxifen i.p for 5 consecutive days. Single spleen cell suspension were obtained 7 days after the first tamoxifen treatment **(A)**. Cells were staned for flow cytometric analysis with Abs against TCR-β, CD4 and STAT3. Representative plots of the analysed data are shown in **(B)**. MFI of STAT3 in the CD4 population is shown in the bar graph **(C)**. Results are shown as mean ± SD of 4 - 5 mice per group. (* P ≤ 0.02; Mann-Whitney Rank Sum Test).

To investigate whether this transient reduction in STAT3 affects the frequencies of TFH-10 and TR1 cell populations in old mice, an intracellular flow cytometric analysis was performed on single spleen cell suspensions of old cre^{+} and cre^{-} STAT3$^{f/f}$ mice and the relative proportions of TFH-10 and TR1 cells within the CD4 T-cell population was analysed. Although there was a transient STAT3 reduction after tamoxifen treatment, the frequency of TFH-10 cells in the CD4 T-cell population was similar in both mouse groups (Fig. 19A, B). The same was found when TR1 cells within the CD4 T-cell population was examined (Fig. 19A, C). These results suggests that a transient STAT3 deletion has no influence on TFH-10 or TR1 accrual in old mice.

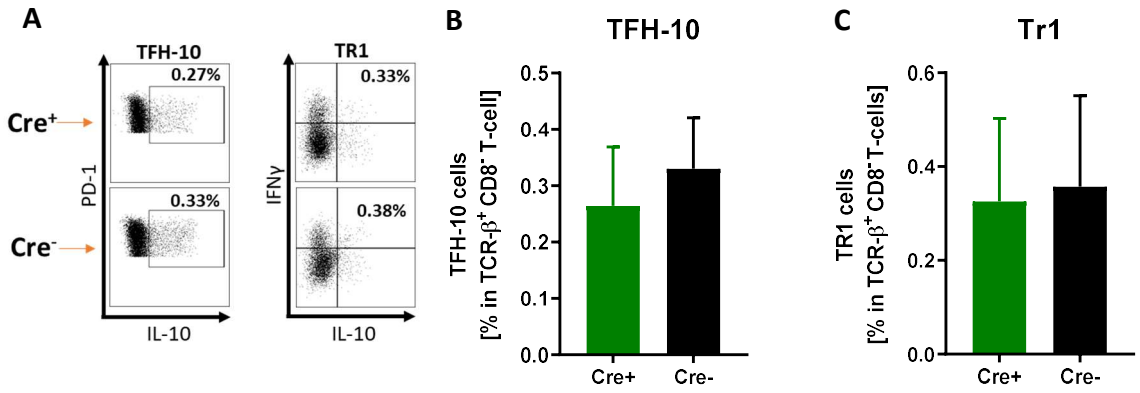

Figure 19. Short term knockdown of STAT3 has no effect on the TFH-10 or TR1 cell population in old mice
Single spleen cells suspension from old STAT3$^{f/f}$ CD4 creER^{T2+} and STAT3$^{f/f}$ CD4 creER^{T2-} mice were stimulated for 5 h with PMA/Ionomycin, blocked with Brefeldin A and labelled for flow cytometry analysis with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10. The Relative frequencies of TFH-10 (see Fig 2E) and TR1 (see Fig 2F) cells in CRE^{+} or CRE^{-} mice are shown in the plots and graphs above **(A—C)**. Results are shown as mean ± SD of 4 - 5 mice per group.

3.2.10 Cytokines other than IL-6 involved in the accrual TFH-10 and TR1 cells with age

Besides IL-6, IL-21 and IL-27 are thought to be inducers of FoxP3^{-} IL-10^{+} CD4 T-cells, particularly TFH-10 and TR1 cells[95,148,154]. To examine whether these cytokines are indeed elevated in old mice, intracellular cytokine staining and serum ELISA were performed in the present thesis. To detect IL-21 production in CD4 T-cells, spleen cells from young and old C57BL/6 mice were stimulated, fluorescently labelled and the relative proportion of IL-21-producing CD4 T-cells was measured. IL-21 production from CD4 T-cells was significantly increased in older mice (Fig. 20A). With the aid of IL-27p28, IL-27 production was also assessed in serum samples collected from the vena cava of young and old mice. A significant increase serum levels of IL-27p28 in older mice was also observed (Fig. 20B). Taken together, these results suggest that in addition to IL-6, IL-21 and IL-27 may also contribute to the increase in FoxP3^{-} IL-10^{+} CD4 T-cells with advancing age.

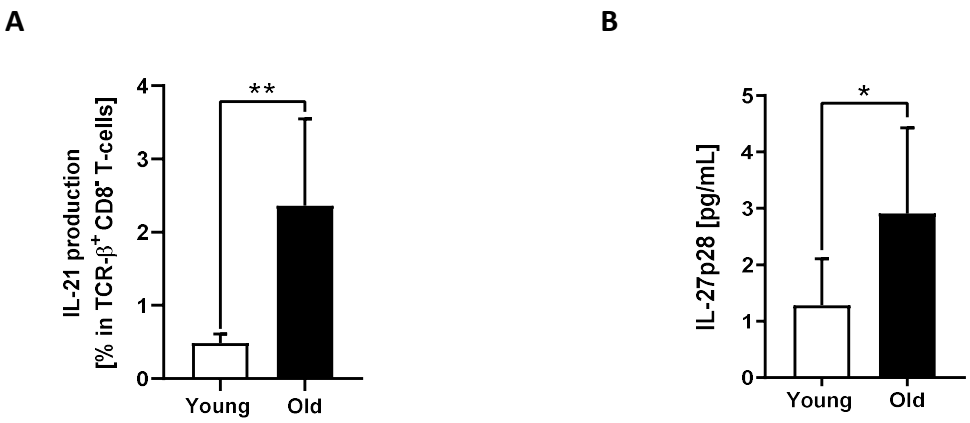

Figure 20. IL-21 and IL-27 levels are increased in old mice
Single spleen cell suspension from C57BL/6 young (6 wks old) and old (17 mths old) mice were stimulated for 5h with PMA/Ionomycin, blocked with Brefeldin A and labelled with antibodies against TCR-β, CD8 and IL-21 for flow cytometric analysis. The relative proportion of IL-21 production in $CD4^+$ T-cells is shown in **(A)**. Basal IL-27 production in serum samples of young (4 mths old) and old (16 mths old) C57BL/6 mice were measured by ELISA. Mice were euthanised, vena cava blood was collected, and IL-27 was measured in serum **(B)**. Flow cytometry data shows the mean ± SD of 4-8 mice per group. (** $P \leq 0.04$; Mann-Whitney Rank Sum Test). ELISA Data are shown as mean ± SD of 16 mice per group (* $P \leq 0.01$; Mann-Whitney Rank Sum Test)

3.2.11 Loss of IL-6 results in reduced IL-21 and IL-27 production in older mice

By using IL-$6^{-/-}$ mice, the effect of IL-6 signalling, on IL-21 and IL-27 production in young and old mice was analysed. Intracellular flow cytometry analysis was performed on spleen cells of young and old C57BL/6 and IL-$6^{-/-}$ mice. Whereas IL-21 production in CD4 T-cells from young C57BL/6 and IL-$6^{-/-}$ mice was similar, IL-21 expression was abrogated in older IL-$6^{-/-}$ mice and a significant difference compared to old C57BL/6 was observed (Fig. 21B). IL-27p28 production in the TR1 population was also investigated in C57BL/6 and IL-$6^{-/-}$ mice. Like IL-21 expression in CD4 T-cells, IL-27p28 production was also significantly reduced in old IL-$6^{-/-}$ mice when compared to their C57BL/6 counterpart. In young mice, there was no difference in IL-27p28 production (Fig. 21D).

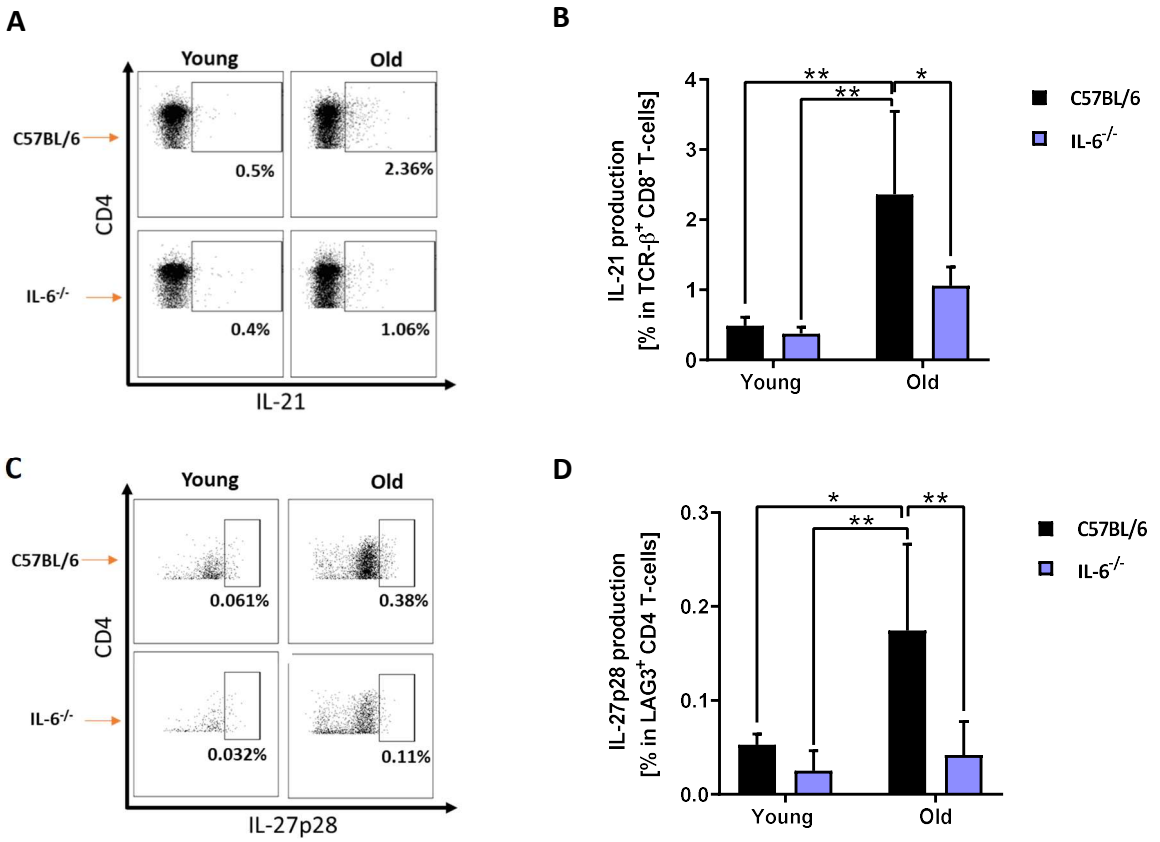

Figure 21. Decreased IL-21 and IL-27p28 production in the absence of IL-6

Single spleen cell suspensions from young (2 mths old) and old (17 mths old) C57BL/6 and IL-6$^{-/-}$ mice were stimulated for 5 h with PMA/Ionomycin and Brefeldin A or for 24 h with LPS and Brefeldin A. Labelled for flow cytometry with Abs against TCR-β, CD8, LAG3, FoxP3 IL-21 and IL-27p28. Representative dot plots are shown in **(A)** and **(C)**. The relative proportion of IL-21 in TCR-β^{+} CD8^{-} (CD4^{+}) and IL-27p28 in the TR1 population is shown in the bar graphs **(B)** and **(D)** respectively. Gate for IL-27p28 is set based on unstimulated controls. Results are shown as mean ± SD of 4 – 8 mice per group and is representative of 1 experiment. (* P ≤ 0.02, ** P ≤ 0.004; Two-way ANOVA with Tukey's multiple comparison)

3.2.12 Loss of IL-21 or IL-27 signalling affects specific FoxP3^{-} regulatory populations

In the present work, TFH-10 cells and TR1 cells are the source of increased IL-10 production by CD4 T-cells in old age. Because IL-21 and IL-27 were identified as factors for the induction or differentiation of these cells respectively[95,148]. IL-21$^{-/-}$ and IL-27Rα$^{-/-}$ mice were used to determine the effect of these cytokines on accrual of TFH-10 and TR1 cells in advancing age. Using intracellular flow cytometric analysis, the proportions of TFH-10 and TR1 cells in CD4 T-cells of young and old C57BL/6 and mutant mice were examined. Young C57BL/6 and IL-21$^{-/-}$ mice had similar frequencies of TFH-10 and TR1 cells whereas in old mice, there was a significant reduction of both cell populations when IL-21 was deleted (Fig. 22A—C). Likewise, old IL-27Rα$^{-/-}$ mice had a significant decrease in the frequency of TR1 cells when compared to old C57BL/6 mice (Fig. 23C). But contrary to IL-21$^{-/-}$ mice, the TFH-10 population in IL-27Rα$^{-/-}$ mice remained unaffected by the loss of IL-27 signalling (Fig. 23B). In young mice, there was

no significant difference in TFH-10 or TR1 frequencies in either group (Fig. 23B, C). These results indicate that IL-21 signalling is not only important for the differentiation of TFH-10 cells but also for TR1 cells whereas IL-27 signalling plays a more specific role in the differentiation of cells to a TR1 phenotype. It also shows that both IL-21 and IL-27 signalling plays a more significant role in accrual of FoxP3-negative IL-10-producing cells in old age.

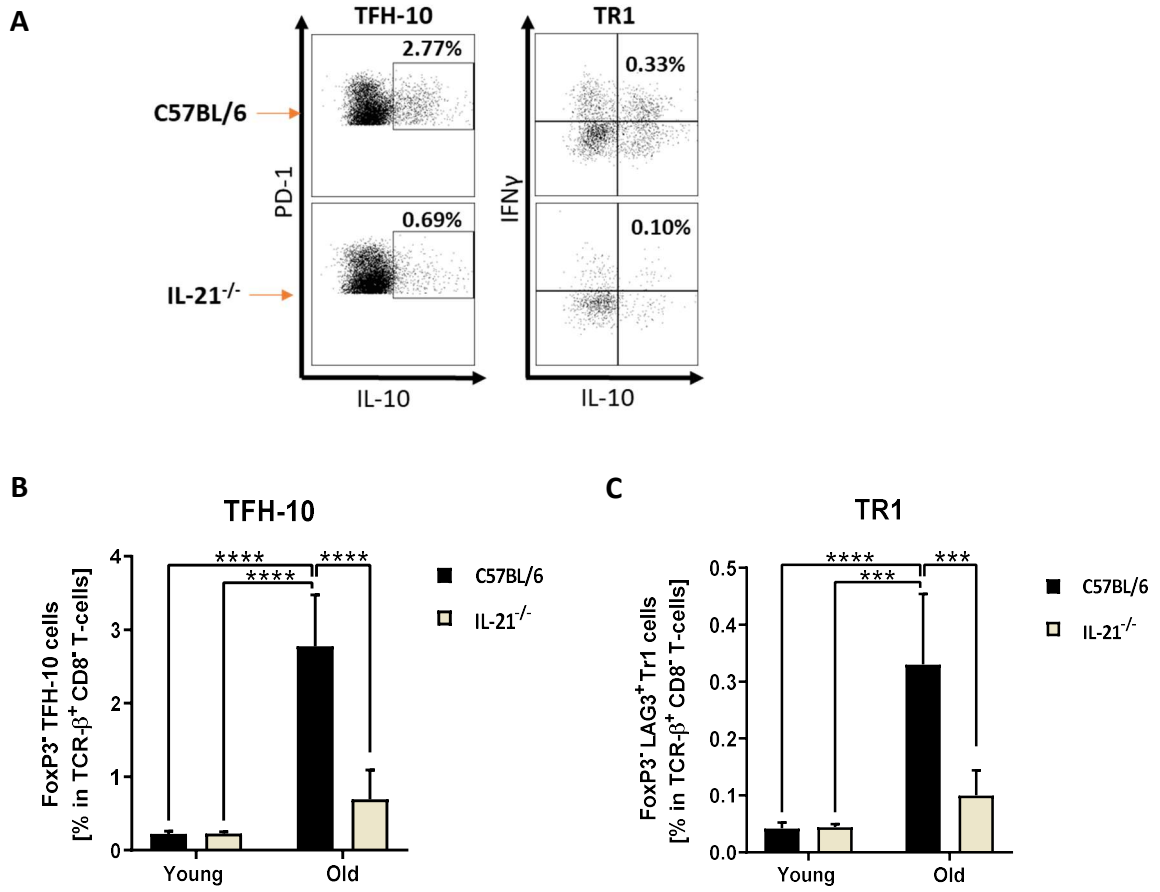

Figure 22. IL-21 deficiency alters TFH-10 and TR1 cell frequencies in old but not young mice
Single spleen cell suspensions from young (2 mths old) and old (17 mths old) C57BL/6 and IL-21$^{-/-}$ mice were stimulated for 5 h with PMA/Ionomycin, blocked with Brefeldin A and labelled with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10 for flow cytometry analysis. The relative proportions of TFH-10- **(A, B)** and TR1-producing cells **(A, C)** in the TCR-β^{+} CD8^{-} (CD4^{+}) T-cells population in the different mouse strains are depicted in the plots and bar charts above. Results are shown as mean ± SD of 4 – 8 mice per group (**P < 0.01 *** P < 0.0003, **** P < 0.0001; Two-way ANOVA with Tukey's multiple comparison).

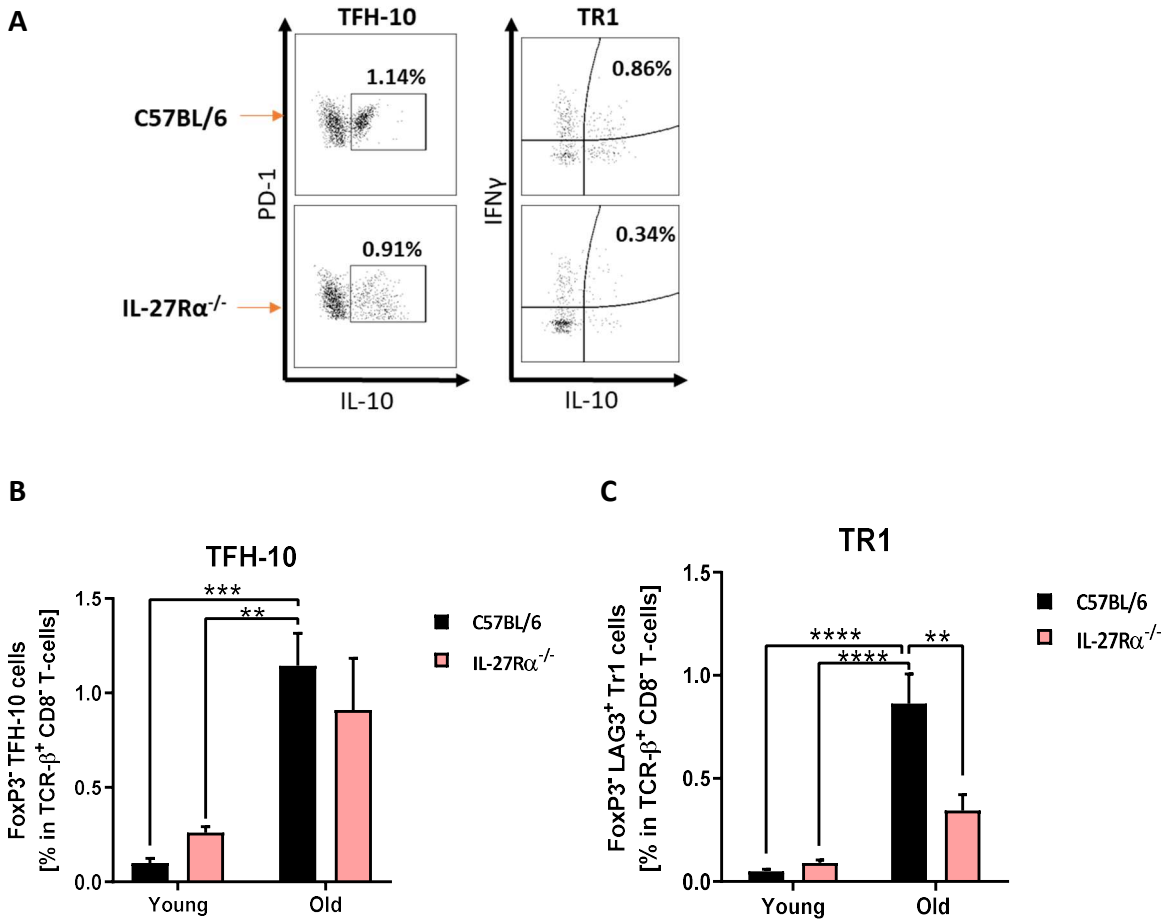

Figure 23. IL-27Rα deficiency alters TR1 but not TFH-10 cell frequencies in old mice
Single spleen cell suspensions of young (2 mths old) and old (14 mths old) C57BL/6 and IL-27Rα$^{-/-}$ mice were stimulated for 5 h with PMA/Ionomycin, blocked with Brefeldin A and labelled with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ and IL-10 for flow cytometry. The relative proportions of TFH-10- **(A, B)** and TR1-producing cells **(A, C)** in the TCR-β^{+} CD8^{-} (CD4^{+}) T-cells population in the different mouse strains are depicted in the plots and bar charts above. Results are shown as mean ± SD of 4 – 8 mice per group and is representative of 2 experiments (**P < 0.01 *** P < 0.0003, **** P < 0.0001; Two-way ANOVA with Tukey's multiple comparison).

3.2.13 Transcription factor expression in the FoxP3-negative CD4 T-cell population is decreased in mutant mice

Recognising that changes in transcription factors activity is a hallmark of aging[2], the relationship between transcription factors and the cytokines IL-6, IL-21 and IL-27 was investigated to understand molecular mechanisms involved in the accumulation of TFH-10 and TR1 cells with age. Two of the transcription factors examined were Early Growth Response Gene 2 (EGR2), described by Okamura et al in FoxP3^{-} regulatory T-cell populations[171], and cMAF, which was identified as one of the strongest candidates for IL-10 induction in CD4 T-cells due to its correlation with *IL10* mRNA expression[172]. Therefore, in the present thesis single spleen cell suspension from young and old C57BL/6, IL-6$^{-/-}$, IL-21$^{-/-}$, or in the case of IL-27R$^{-/-}$ only old mice, were stimulated and stained for EGR2 or cMAF in the TFH-10 and TR1

populations. As expected, both transcription factors were increasingly expressed in TFH-10 and TR1 cells in old C57BL/6 mice when compared to younger mice (Fig. 24A—D).

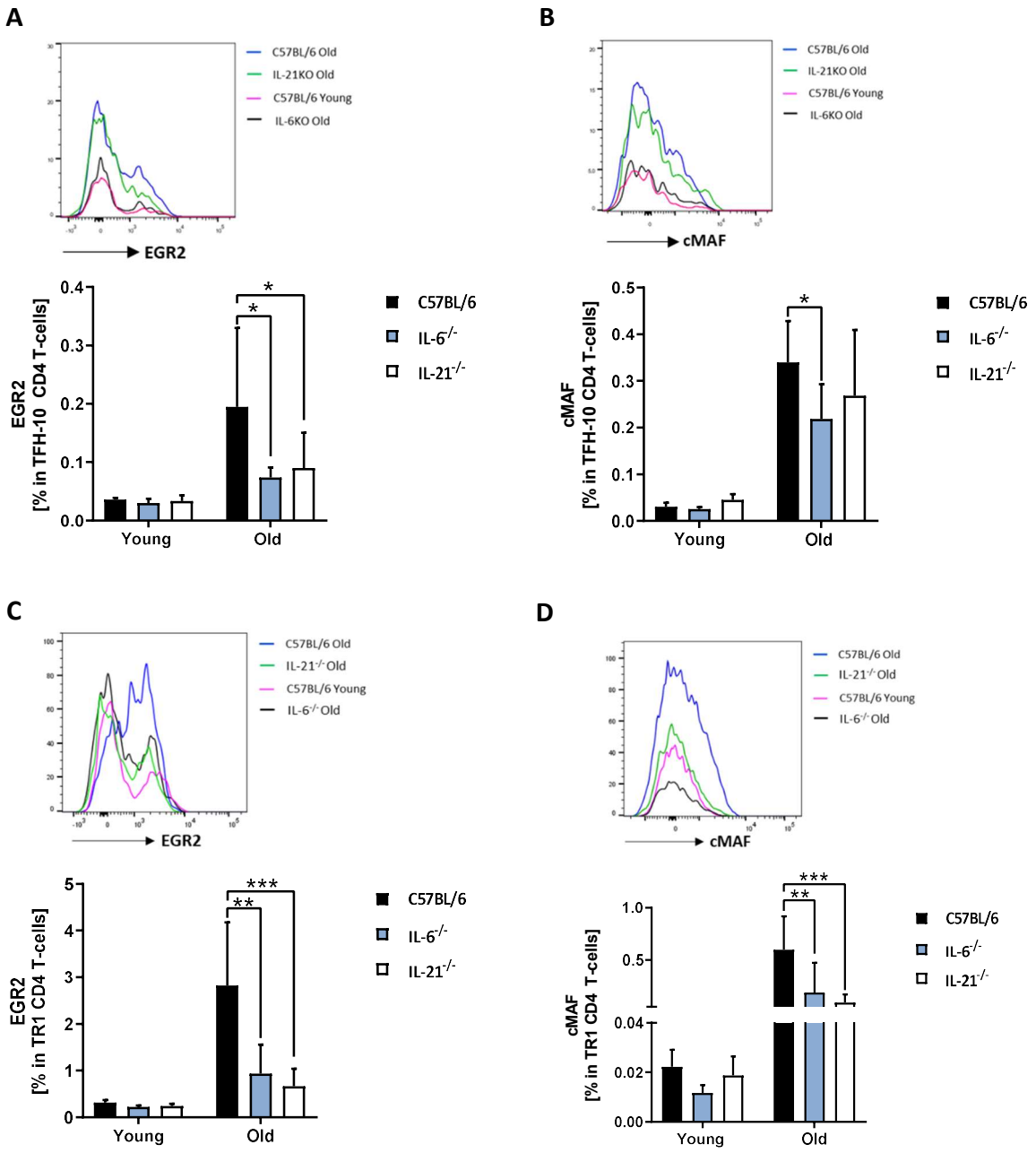

Figure 24. Decreased expression of EGR2 and cMAF in young and old IL-6$^{-/-}$ and IL-21$^{-/-}$ mice
Single spleen cell suspensions young (2 mths old) and old (14 mths old) C57BL/6, IL-6$^{-/-}$ and IL-21$^{-/-}$ mice were stimulated for 5 h with PMA/Ionomycin and Brefeldin A, then stained for flow cytometry with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ, IL-10, cMAF and EGR2. The relative proportions of EGR2 **(A & C)** and cMAF **(B & D)** expression within the TFH-10 and TR1 cells in TCR-β^{+} CD8^{-} (CD4^{+}) T-cells population of the different mouse strains are depicted in the bar charts above. Results are shown as mean ± SD of 4 – 8 mice per group (*P ≤ 0.04, **P ≤ 0.003, ***P ≤ 0.0004; Two-way ANOVA with Tukey's multiple comparison).

The expression of EGR2 in TFH-10 cells was also decreased in IL-6$^{-/-}$ and IL-21$^{-/-}$ mice when compared to old C57BL/6 mice (Fig. 24A). cMAF was also reduced in TFH-10 cells from old mutant mice but the difference was only significant when old IL-6$^{-/-}$ and C57BL/6 mice were

compared (Fig. 24B). When compared to old C57BL/6 mice, the expression of both EGR2 and cMAF in TR1 cells was significantly reduced in old IL-6$^{-/-}$ and IL-21$^{-/-}$ mice (Fig. 24 C, D).

When old IL-27Rα$^{-/-}$ mice were compared to old C57BL/6 mice, EGR2 was significantly reduced in TR1 but not in TFH-10 cell while cMAF expression was significantly impaired in TFH-10 but not in TR1 cells from mutant mice (Fig. 25A—D). Hence, IL-27 appears to selectively support the expression of EGR2 and cMAF in TR1 and TFH-10 cells respectively. Taken together, these results suggest that an interplay between cytokine signalling and transcription factor expression leads to the development of FoxP3-negative regulatory CD4 T-cell subsets during aging.

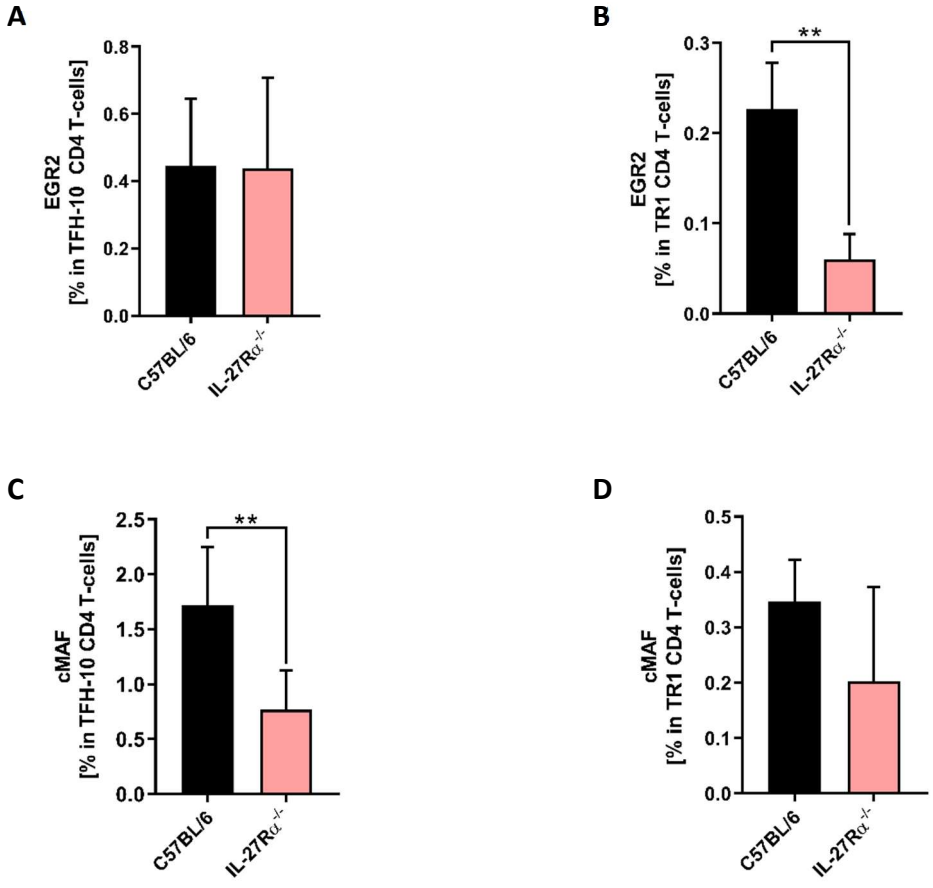

Figure 25. Decreased expression of transcription factors in old IL-27Rα$^{-/-}$ mice
Single spleen cell suspensions from young (5 mths old) and old (13 mths old) C57BL/6, and IL-27Rα$^{-/-}$ mice were stimulated for 5 h with PMA/Ionomycin and Brefeldin A then stained for flow cytometry with Abs against TCR-β, CD8, FoxP3, Lag3, CXCR5, PD-1, IFNγ, IL-10, cMAF and EGR2. The relative proportions of EGR2 **(A & C)** and cMAF **(B & D)** expression within the TFH-10 and TR1 cells in TCR-β^{+} CD8^{-} (CD4^{+}) T-cells population of the different mice strains are depicted in the bar charts above. Results are shown as mean ± SD of 4 – 8 mice per group (**P ≤ 0.002; Two-way ANOVA with Tukey's multiple comparison).

3.2.14 Comparative in vitro study of the suppressive effect of FoxP3-negative regulatory T-cells

To investigate the suppressive activity of TFH-10 or TR1 cells, Lag3-negative effector T-cells (T_{eff}) sorted from young FoxP3^{DTR} mice were labelled with CellTrace^{TM} violet and co-cultured *in vitro* under T-cell receptor stimulation (anti-CD3/CD28) with FoxP3-positive Tregs, FoxP3-negative Lag3-positive TFH-10 and TR1 cells from older FoxP3^{DTR} mice in a 1:1 ratio. After 72 hours, proliferation of T_{eff} with or without the addition of regulatory cells was analysed by flow cytometry. During proliferation, each peak depicts the dye dilution between generations of T-cells (Fig. 26). Addition of TFH-10 cells significantly decrease the proliferation of T_{eff} by 20%. To determine if the suppressive effect of TFH-10 cells was a result of IL-10 production, an IL-10 receptor block (αIL-10R) was added to another culture of T_{eff} and TFH-10. The addition of the αIL-10R reversed the suppressive effect of TFH-10 cells and the proliferation of Teff was similar to Teff without TFH-10 cells (Fig. 26A, B).

The effect TR1 cells have on inhibiting proliferation was also tested and a difference of roughly 9% was observed between the proliferating T_{eff} and T_{eff} with TR1 cells (Fig. 26C, D). As an internal experimental control, Tregs were also sorted and used to inhibit the proliferation of T_{eff}. A marked suppression of T_{eff}, by 41.8%, was observed (Fig. S2).

To mimic antigen-specific effects during infection with *Mtb*, bone marrow derived dendritic cells from young C57BL/6 mice were co-cultured with CellTrace^{TM} labelled T_{eff}, TFH-10 or TR1 CD4 T-cells from old P25 TCR transgenic mice in the presence of P25 peptide antigen as a stimulant. On day 4, proliferation of T_{eff} was measured by flow cytometry. Although not significant, proliferation was inhibited when TFH-10 or TR1 cells were added to the culture, with TFH-10 cells having a stronger inhibitory effect on proliferating effector T-cells in comparison the TR1 cells. When αIL-10R was added to the culture of T_{eff} with TFH-10 or TR1 cells, a slight increase in proliferation was observed (Fig. S3). The data confirm the suppressive activity of IL-10 and suggest that TFH-10 plays a more important role as a regulatory cell than TR1 cells. A limitation of this experiment was the lack of regulatory cells from young mice to draw comparisons with the results from older mice. This is because young mice produce significantly fewer TFH-10 and TR1 cells which would mean that an incredibly large number of mice would have had to be sacrificed to obtain enough cells for such an experiment. An interesting question would be whether TFH-10 and TR1 cells from young mice are as

suppressive as those from old mice and whether it is only because of their low numbers in young mice that we do not detect any effects of these cells on the immune response.

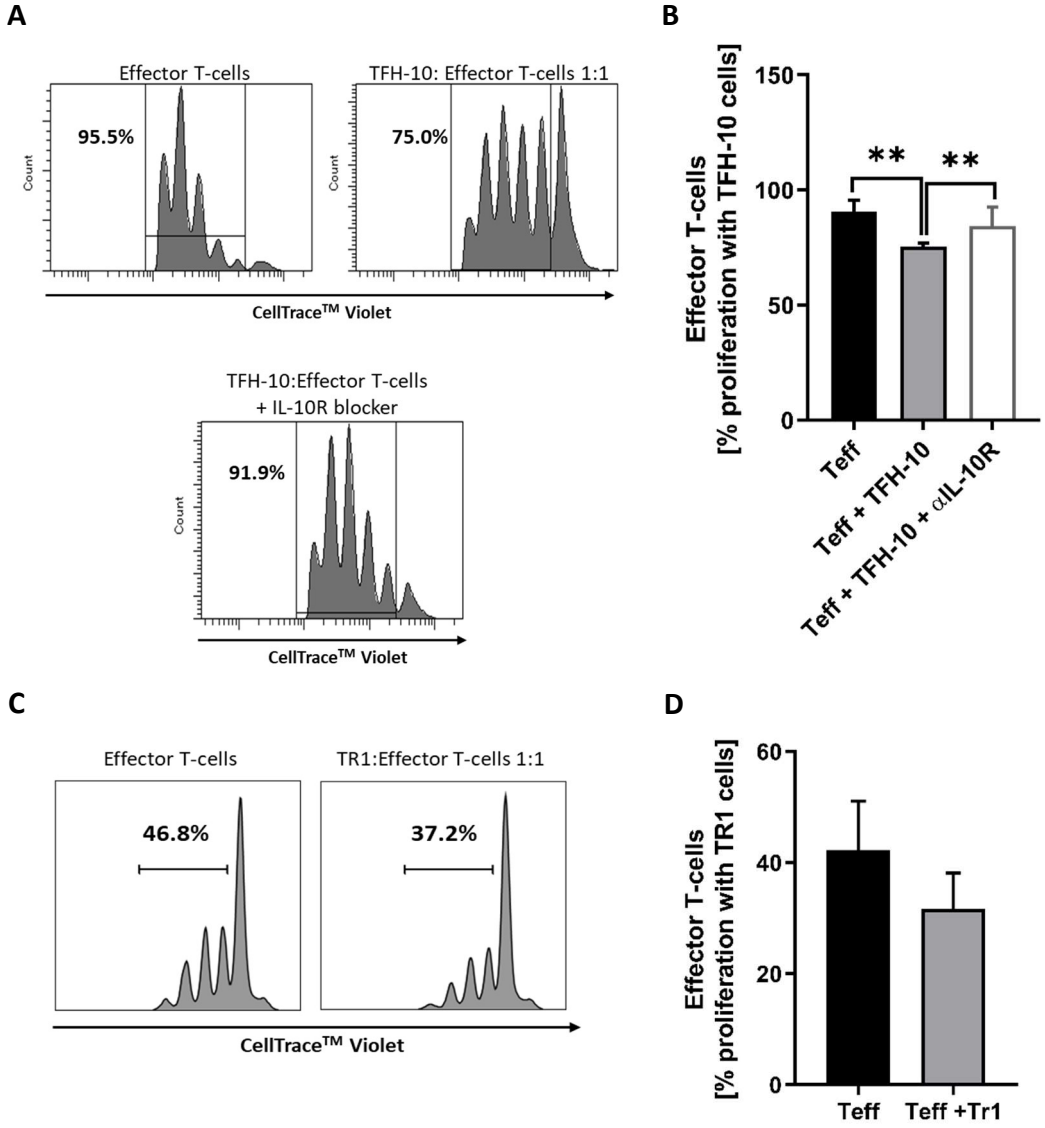

Figure 26. TFH-10 and TR1 cells suppress the proliferation of effector T-cells in an antigen-unspecific manner

The suppressive effects of the different regulatory T-cells populations were assessed using flow cytometry. Single spleen cell suspensions from young and old FoxP3^{DTR} mice were enriched for CD4^{+} T-cells by magnetic cell separation. Cells from younger mice where subsequently sorted into Lag3-negative effector T-cells, labelled with CellTrace^{TM} Violet and activated with CD3/CD28. Cells from older mice were sorted in to 3 distinct regulatory T-cell populations: FoxP3-positive Tregs, FoxP3-negative Lag3-positive TFH or TR1 cells and co-cultured for 72 hrs with the CellTrace^{TM} Violet labelled effector T-cells from young FoxP3^{DTR} at a ratio of 1:1. Resul**t** shown in **(A, C)** is the dilution of the proliferation dye in effector T-cells. Bar graph in **(B, D)** shows percentage decrease of proliferation of effector cells after co-culture with regulatory T-cell. Bar graph in **(B)** also shows the effect of IL-10 signalling blockade on TFH-10 cells and proliferation

The increased expression of IL-6 with age increases the population of regulatory FoxP3$^-$ IL-10$^+$ CD4 T-cells that could impair immune responses. How IL-6 leads to the increase in this cell population is still unclear, as phosphorylation of STAT proteins at the tyrosine residue after classical or trans-signalling did not lead to an increase in regulatory FoxP3$^-$ TFH-10 or TR1 cells in old mice. However, the loss of IL-6 signalling and the subsequent loss of its downstream cytokine targets such as IL-21 or IL-27 led to a significant reduction in transcription factors involved in the accrual of TFH-10 and TR1 cells. Furthermore, the results in the present thesis showed that IL-10 production by TFH-10 and TR1 cells suppresses T-cell proliferation in an antigen-dependent and -independent manner.

Increased production of regulatory cells is shown to be detrimental during infection and mouse models highlight the effects of Treg expansion in *Mtb* infections. The present thesis aims to find out whether the age-dependent increase of FoxP3$^-$ regulatory cells inadvertently increases susceptibility to *Mtb* infection.

3.3 Infection and aging in experimental TB

Although TB affects individuals of all ages, the relative rates of new cases and deaths amongst the elderly is increased[17]. Reduced naïve T-cell output by the thymus, defined by their reduced capacity for cell proliferation as well as increased numbers of antigen-specific Treg are some of the most consistent reported effects of aging on the immune system[90]. The present work shows an increase in the population of FoxP3$^-$ regulatory CD4 T-cells with age. The extent to which the increase of FoxP3$^-$ regulatory CD4 T-cells is relevant to the susceptibility and progression of *Mtb* infection is of interest. In line with these findings, young and old C57BL/6 and mice with altered IL-27 signalling was employed in the present thesis to profile and understand inflammatory and regulatory responses in geriatric pulmonary tuberculosis.

3.3.1 Effect of aging on progression of *Mtb* infection in young and old wild-type mice

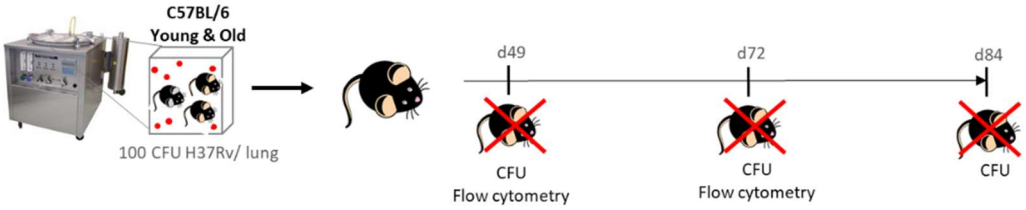

Figure 27. Schematic of the *in vivo Mtb* experiments
At the indicated time points after aerosol infection with 100 CFU *Mtb* H37Rv,mice were sacrificed and bacterial load and immune responses in the lungs were measured and analysed

3.3.1.1 Increased CFU counts in older mice

In sections 3.1.2 and 3.1.3 of the present thesis, FoxP3$^-$ CD4 T-cells were unmasked as major IL-10 producers in aged mice. With this information, young and old C57BL/6 mice were infected with 100 CFU *Mtb*. The bacterial load in the lungs of these mice were investigated and the effects of aging on the course of infection at 49-, 72- and 84-days post infection were assessed. Younger mice were better able to control the infection (Fig. 28). Although no significant difference in bacterial loads in the lungs was found 49 days after the initial aerosol infection, older mice still showed a tendency to have higher bacterial burdens than younger mice at this time point. As infection progressed, a reduction in bacterial burden by approximately 0.5 $\log_{10}$ units was recorded after CFU counts on days 72-and 84 in the lungs of younger mice. A significant reduction in bacterial load was also observed when young and old mice were compared on day 72- and day 84. In older mice, the bacterial load over the course of infection from day 49 through till day 84 remained constant (Fig. 28). This lack of reduction in bacterial burden in older mice points to an inability of these mice to control the infection.

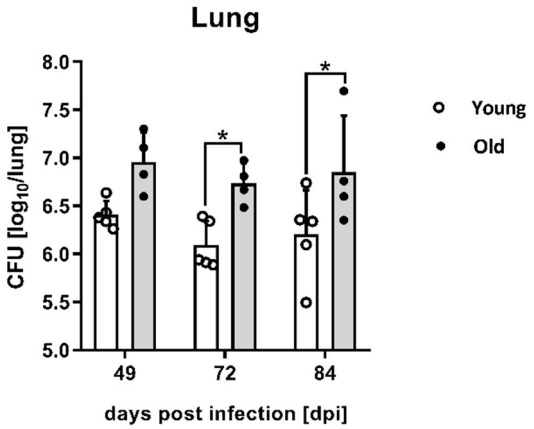

Figure 28. Aged mice are less successful at controlling *Mtb* infection
After aerosol infection of young (4 mths old) and old (24 mths old) C57BL/6 mice with 100 CFU *Mtb*. A colony enumeration assay in lung tissue was performed over the course of infection at different time points post infection. Results are shown as mean ± SD of 4 – 5 mice per group (*P ≤ 0.04; two-way ANOVA with Sidak's multiple comparison).

3.3.1.2 Increased infiltration of FoxP3-negative regulatory T-cells into the lung tissue of older mice during Mtb infection

With the knowledge that the bacterial burden is increased in older mice, close attention was paid to the CD4 T-cells response. It is known that *Mtb* infection leads to increased infiltration of pathogen-specific Tregs to the site of infection[173,174] which could delay T_{eff} infiltration and lead to increased bacterial load. Consequently, the present thesis investigated the infiltration of other regulatory immune cells at the infection site on day 49 and day 72 post infection. This was done by preparing single cell suspensions from lung tissue of young and old C57BL/6 mice, stimulating them with α-CD3/CD28 antibodies and staining TFH-10 and TR1 cells for flow cytometry. At both time points, significantly increased frequencies of circulating TFH-10 (Fig. 29A) as well as TR1 (Fig. 29B) cells were detected in lung tissue of older mice.

As presented in this thesis, FoxP3$^-$ regulatory CD4 T-cells are already increased in lymphoid organs of uninfected old mice. Because infiltration of uninfected lungs with immune cells is absent, confirmation of infection induced increase in TFH-10 and TR1 cells was also performed using single spleen cell suspension of age matched *Mtb* infected and uninfected C57BL/6 mice. For both cell subsets, there was a significant increase in TFH-10 and TR1 frequencies in the spleen of older mice after *Mtb* infection in comparison to older uninfected mice (Fig. 29C, D). However, in younger mice, frequencies of both cell populations were similar in both infected and uninfected cohorts (Fig. 29C, D). This result suggests that in addition to Tregs, FoxP3-negative regulatory TFH-10 and TR1 cells are also recruited to the site of *Mtb* infection and age affects the frequency of regulatory cells infiltrating lymphoid organs and infected tissue.

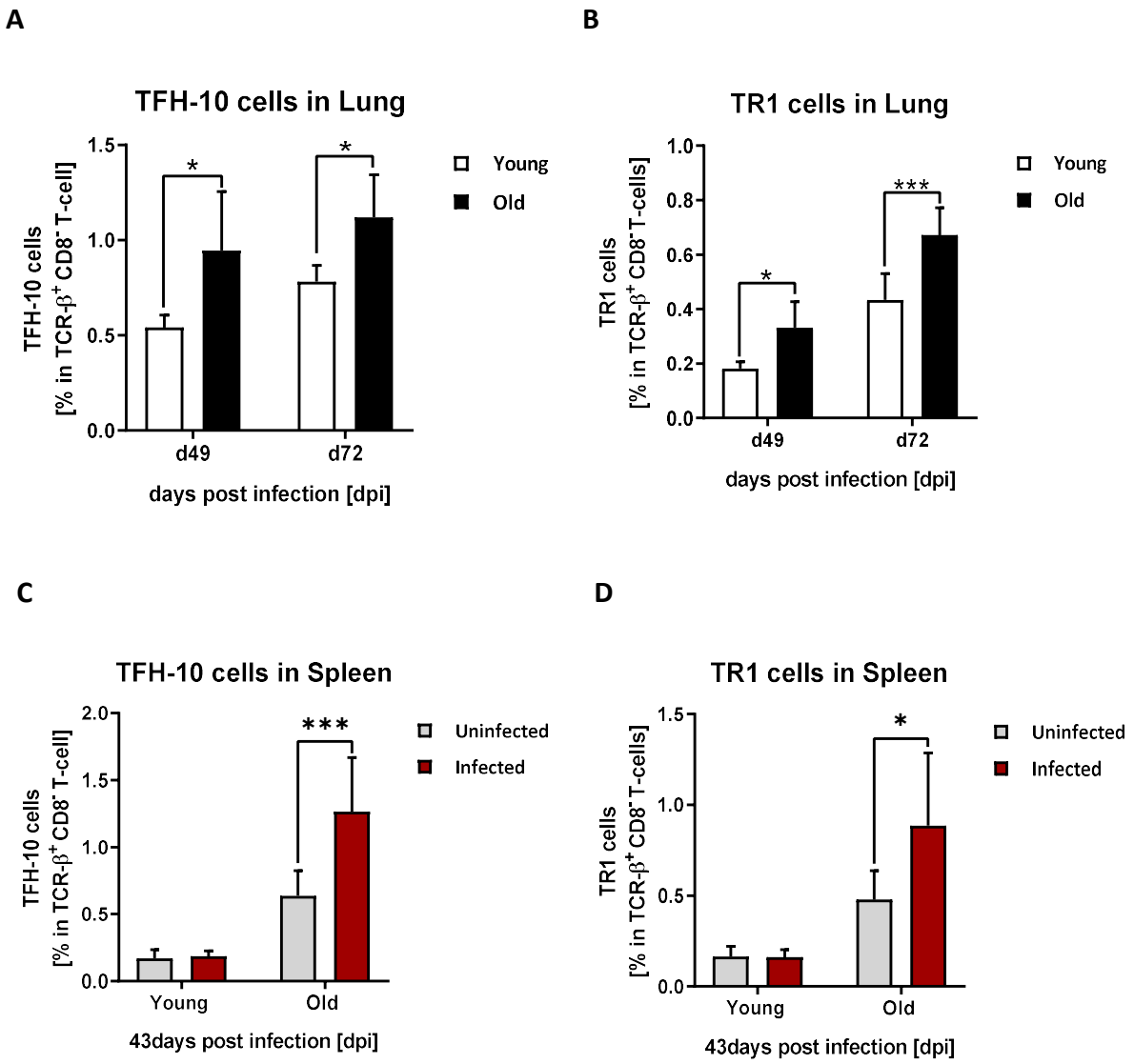

Figure 29. *Mtb* infected lungs of older mice show an increase in FoxP3⁻ regulatory CD4 T-cells
Young and old C57BL/6 mice were divided into infected and uninfected cohorts. Aerosol infection was performed with 100 CFU *Mtb*. At the indicated time points after infection , single cells suspension from lung tissue and spleens of both infected and uninfected mice were stimulated for 5 h with anti-CD3/CD28 and labelled with Abs against CD4, CXCR5, PD-1, LAG3, FoxP3, IL-10 and IFNγ. The bar chart above show the relative frequencies of TFH-10 cells within the FoxP3⁻ PD-1/CXCR5⁺ IL-10⁺ CD4 T-populations **(A, C)** and TR1 within the FoxP3⁻ LAG3⁺ IL-10/IFNγ⁺ CD4 T-populations **(B, D)**. Results are shown as mean ± SD of 4 - 5 mice per group. (*P ≤ 0.04, **P≤ 0;001 two-way ANOVA with Sidak's multiple comparison).

3.3.1.3 After Mtb infection, recruitment of ESAT-6-specific CD4 T-cells is unaffected by age

Infections with *Mtb* lead to the development of a complex immune response with a mixed population of antigen-specific CD4 T-cells that are critical for clearing *Mtb* bacteria. Some of the CD4 populations include TH1 and even suppressive regulatory CD4 T-cells.

ESAT-6, a secreted *Mtb* antigen, elicits a strong TH1 immune response that provides the best protection compared to other *Mtb* antigens, as it has immunodominant properties and is recognised by the immune cells throughout the infection[175,176]. In one report, despite adoptive transfer of ESAT-6-specific CD4 T-cells prior to infection, there was a delay in protective

immune response, mainly due to the inactivity of the transferred cells[177], potentially indicating regulatory cell action on the transferred cells.

In the present thesis, ESAT-6-specific CD4 T-cell frequencies and numbers were measured after aerosol infection with 100 CFU *Mtb* in C57BL/6 mice to assess the impact of the age-related TFH-10 and TR1 cell increase on the antigen-specific immune response to *Mtb*. At day 49 and day 72, single cell suspension from infected lungs of young and old mice were stimulated with ESAT-6 and CD4 T-cells were subsequently stained with an MHC-II tetramer I-A^b $ESAT6_{(4-17)}$ (Fig. 30A—C). Although no significant difference was observed in the frequencies of ESAT-6-specific cells on day 49 or day 72, a 2-fold increase in the percentage of antigen-specific CD4 T-cells was observed as infection progressed in both young and old mice (Fig. 30A, B). When the absolute number of antigen-specific CD4 T-cells were analysed, no significant difference between young and old mice was observed at either timepoint of analysis (Fig. 30C), although the total number tended to decrease in older mice.

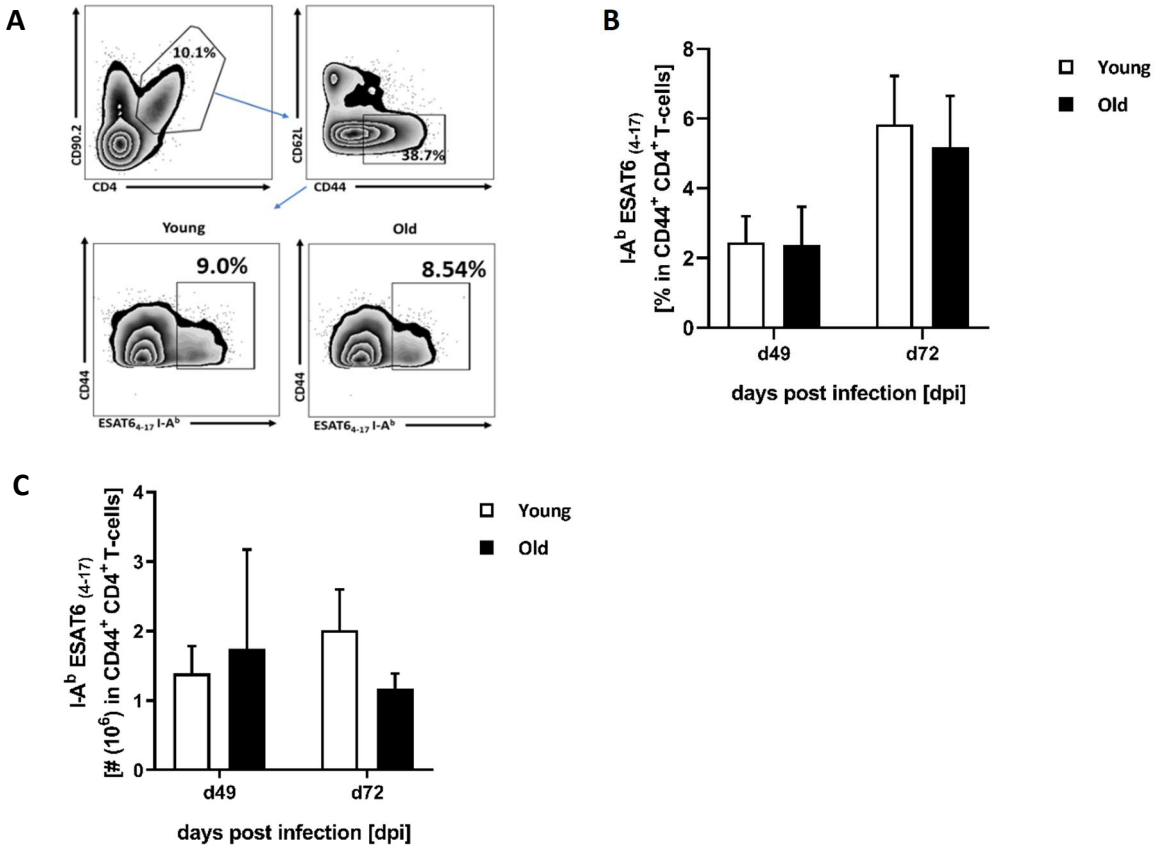

Figure 30. Expansion of antigen-specific CD4 T-cells in *Mtb*-infected lungs of young and old mice.
After aerosol infection of young and old C57BL/6 mice with 100 CFU of *Mtb*, single cells suspension from the lungs were obtained at the indicated time, stimulated for 1 h 15 mins with ESAT-6 and labelled with Abs against CD90.2, CD4, CD44, CD62L and $ESAT6_{(4-17)}$ I-A^b MHC II tetramer for flow cytometric analysis. Density plots show the relative frequencies **(A)** while bar chart shows relative frequencies **(B)** and absolute cell numbers **(C)** of activated $ESAT6_{(4-17)}$ I-A^b specific antigen in CD4 ($CD44^+$ $CD62L^-$) T-cells. Results are shown as mean ± SD of 4 - 5 mice per group.

3.3.1.4 Increased induction of T-cell response in old mice infected with Mtb

TNF-, IFNγ- and IL-2-producing multifunctional T-cells (MuFTs) and IL-17-secreting TH17-cells have been shown to be protective immune responses to *Mtb* infections[178,179] and correspondingly represent some of the cytokines that are increased in during inflammaging[75,105]. However, since the ability to control *Mtb* growth is reduced in older mice (Fig. 28), flow cytometric experiments were performed after polyclonal (α-CD3/CD28) or antigen-specific (STCF) stimulation of single cell suspensions of infected lung cells from young and old mice to determine whether the age-related increase in FoxP3$^-$ regulatory CD4 T-cells affects the production MuFTs and TH17-cells.

Mice were infected with 100 CFU of *Mtb* and at the indicated timepoints after infection, single cell suspensions from lung tissue were obtained and labelled for flow cytometry. After polyclonal T-cell stimulation, there was no significant difference in the frequencies of MuFTs between young and old mice at the earlier timepoint of infection, but by day 72, older mice had significantly increased frequency of MuFTs (Fig. 31B). When an STCF re-stimulation was performed, older mice still had increased frequencies of MuFTs than younger mice, although a significant difference in cytokine production was recorded at day 49 (Fig .31D). In addition, when the frequency of TH17-cells was investigated, it was also significantly increased at day 49 in older mice. As the infection progressed, IL-17A production from TH17-cells in both young and old mice decreased under polyclonal T-cell or antigen-specific re-stimulatory conditions (Fig. 31C, E). IL-17 production was significantly higher in older mice than in young mice
(Fig. 31C, E). The results indicate that with aging, the release of inflammatory cytokines increases during *Mtb* infection, without this being influenced by the increased number of Foxp3$^-$ regulatory CD4 T-cells. While this would suggest a better prognosis for older mice, this is not the case as older mice had a higher bacterial load.

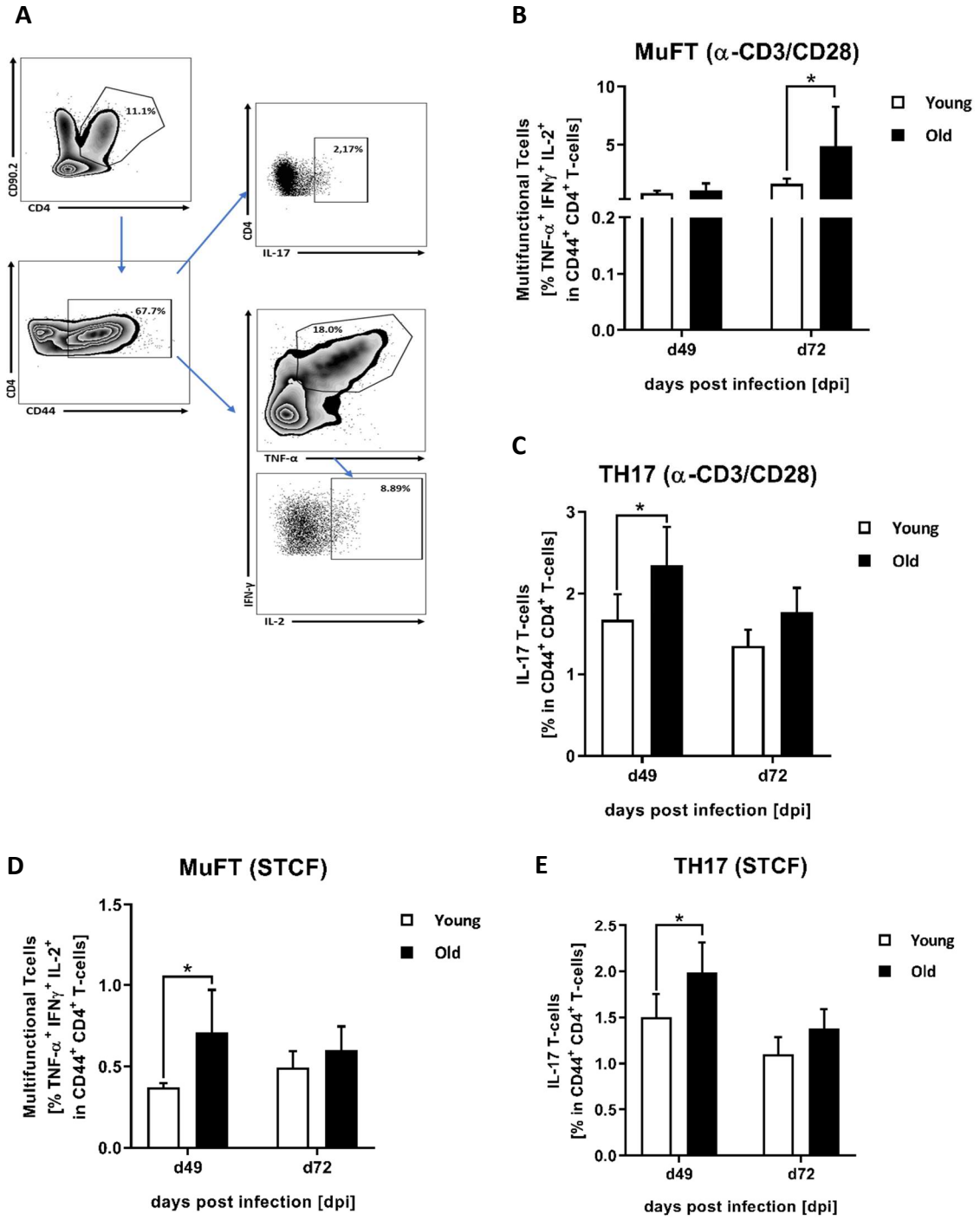

Figure 31. The induction of MuFTs and TH17-cells is increased in older mice after *Mtb* infection
Young and old C57BL/6 mice were infected via aerosol with 100 CFU *Mtb*. At the indicated time points after infection, single cell suspensions from the lungs was prepared, stimulated for 5 h with anti-CD3/CD28 or STCF and labelled with Abs against CD90.2, CD4, CD44, IL-17, IL-2, TNF and IFNγ. Density plots show the gating strategy **(A)** and bar chart above depicts the relative frequencies of MuFTs **(B & D)** and Th17-cells **(C& E)** within the $CD4^+$ $CD44^+$ T-cell population. Data are shown as mean ± SD of 4 - 5 mice per group (*P ≤ 0.01, two-way ANOVA with Sidak's multiple comparison).

3.3.2 Effects of aging on the outcome of *Mtb* infection in IL-27Rα$^{-/-}$ mice

3.3.2.1 Effective IL-27 signalling is important for controlling of bacterial burden in older mice

Regarding *Mtb*, studies show that young mice deficient in IL-27Rα have significantly lower bacterial burden and increased number of inflammatory immune cells that localise to TB granulomas[178,180,181]. Furthermore, work in this thesis shows increased IL-27 in serum of old mice, and thus, increased TR1 cell frequencies. Because *Mtb* infection increases the number of infiltrating regulatory cells at the site of infection, which have adverse effects on bacterial clearance, the work in this thesis sought to understand the possibly detrimental role TR1 cells play in TB progression in old age.

C57BL/6 and IL-27Rα$^{-/-}$ mice were used to evaluate the effect of the loss of TR1 cells on bacterial burden in young and old mice. Accordingly, mice were infected with 100 CFU *Mtb* and bacterial loads on day 21 and day 43 post infection. were determined (Fig. 32). At day 21 post-infection, no significant difference was observed between CFUs counts in the lungs of young C57BL/6 and IL-27Rα$^{-/-}$ mice. However, the bacterial load in old IL-27Rα$^{-/-}$ mice was already significantly higher than in old C57BL/6 mice. As infection progressed to day 43 bacterial load in young IL-27Rα$^{-/-}$ mice decreased by approximately 1 Log compared to young C57BL/6 mice. In contrast, the bacterial burden in old IL-27Rα$^{-/-}$ mice significantly increased (approx. 1.2-fold increase) when compared to old C57BL/6 wildtype mice. These data suggest that IL-27 plays an important protective role in TB during aging, whereas it has a detrimental effect at young age.

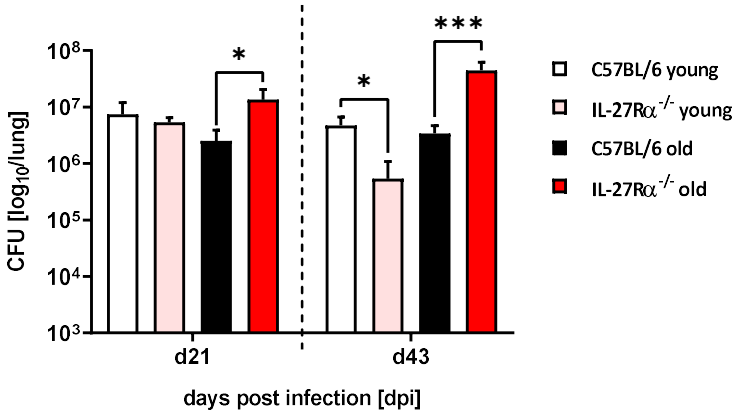

Figure 32. Loss the IL-27Rα leads to an increase in bacterial burden in older mice
After aerosol infection of young (4 mths old) and old (14 mths old) C57BL/6 and IL-27Rα$^{-/-}$ mice with 100 CFU *Mtb*, colony enumeration assay of lung tissue was performed over the course of infection at different time points (21 and 43) post infection. Data are shown as mean ± SD of 4 – 5 mice per group (*P ≤ 0.04, ***P ≤ 0.001; two-way ANOVA with Sidak's multiple comparison).

3.3.2.2 *Increase in TR1 cell frequencies during Mtb infection does not occur when IL-27 signalling is lost*

So far, the present thesis revealed that, when IL-27 signalling is lost, there is a marked reduction in the frequency of TR1 cells with age. Additionally, the loss of IL-27 signalling does not affect the TFH-10 cell population in older mice. Hence, the effect of IL-27Rα deficiency on the frequencies of TR1 cells in the context of an *Mtb* infection was investigated. Young and old C57BL/6 and IL-27Rα$^{-/-}$ mice were infected via aerosol with 100 CFU *Mtb*. At the indicated timepoints post infection, single cell suspension of lung tissue was obtained, stimulated with anti-CD3/CD28 antibodies and fluorescently for flow cytometry in order to determine the relative proportion of TR1 cells in the FoxP3^{-} CD4 T-cell population (Fig. 33) At day 21 post-infection, no significant difference in the frequency of TR1 cell was observed between all mouse groups. On day 43, however, TR1 cells increased in young and old C57BL/6 mice, whereas in the IL-27Rα$^{-/-}$ mice the TR1 frequency remained similar to day 21, with a slight decrease in TR1 cell frequency in young IL-27Rα$^{-/-}$ mice. There was indeed a significant difference in TR1 cell frequency in young IL-27Rα$^{-/-}$ mice compared to young C57BL/6 mice at day 43 post-infection. The same cannot be said when comparing the old cohorts because although TR1 frequency was lower in old IL-27Rα$^{-/-}$ mice compared to old C57BL/6 mice, this increase was not significant.

This result confirms what others have shown that during experimental TB, less infiltration of TR1 cells occurs when IL-27 signalling is disrupted[178].

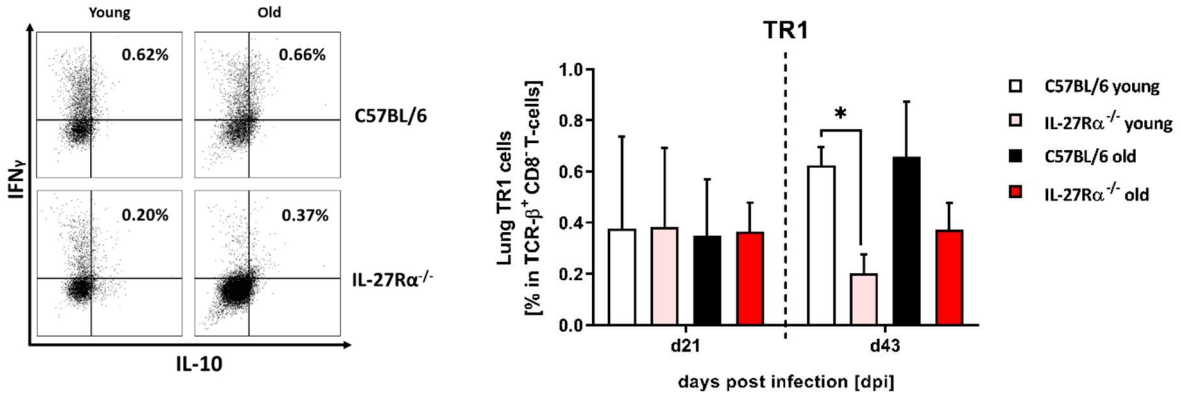

Figure 33. Loss of IL-27 signalling affects TR1 cell frequencies

Young (4 mths old) and old (14 mths old) C57BL/6 and IL-27Rα$^{-/-}$ mice were infected via aerosol with 100 CFU *Mtb*. At the indicated time points after infection, single cells suspension from the lungs were prepared, stimulated for 5 h with anti-CD3/CD28 and labelled with Abs against CD4, CXCR5, PD-1, LAG3, FoxP3, IL-10 and IFNγ. The dot plots and bar chart above show the relative frequencies of TR1 cells within the FoxP3^{-} LAG3^{+} IL-10/IFNγ^{+} CD4 T-cells. Result is shown as mean ± SD of 4 - 5 mice per group, (*P ≤ 0.01; two-way ANOVA with Sidak's multiple comparison).

3.3.2.3 Loss of IL-27 signalling leads to increased frequencies of antigen specific T-cell in old mice at late stages of Mtb infection

In previous experiments in this thesis, no differences in the recruitment of antigen-specific ESAT-6 CD4 T-cell to the lungs of young and old C57BL/6 mice was found. To investigate the effect of a loss in IL-27 signalling on the recruitment of these important CD4 T-cells, young and old C57BL/6 and IL-27Rα$^{-/-}$ mice were analysed 21- and 43 days after infection with 100 CFU of *Mtb*. Single cell suspension from the lungs were obtained, stimulated with anti-CD3/CD28 antibodies, and the ESAT-6-specific CD4 T-cells were determined by flow cytometry. As previously observed, in C57BL/6 mice, the recruitment of ESAT-6-specific CD4 T-cells was similar in young and old mice at both time points, whereas at day 21, young IL-27Rα$^{-/-}$ mice had significantly higher frequencies of the antigen-specific CD4 T-cell population compared to old IL-27Rα$^{-/-}$ mice. By day 43 however, the frequency of antigen-specific CD4 T-cells in young IL-27Rα$^{-/-}$ mice decreased. In contrast, this cell population was found to be significantly increased in old IL-27Rα$^{-/-}$ mice when compared to all the other mouse groups (Fig. 34). This result suggests that IL-27 signalling hampers the recruitment of ESAT-6 antigen-specific cells to the site of infection. In young mice, this recruitment occurs at early stages while in older mice at a later phase of *Mtb* infection.

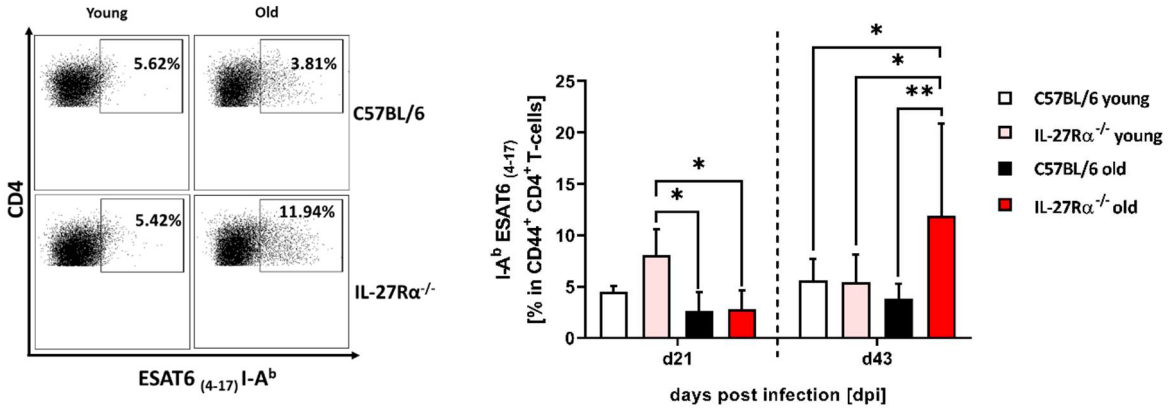

Figure 34. Loss of IL-27Rα promotes an expansion of antigen-specific T-cells in older mice late in infection
Young (4 mths old) and old (14 mths old) C57BL/6 and IL-27Rα$^{-/-}$ mice were infected via aerosol with 100 CFU *Mtb*. At the indicated time points post infection, single cells suspension from the lungs were obtained, stimulated for 1h 15 mins with ESAT-6 antigen and labelled with Abs against CD90.2, CD4, CD44, CD62L and ESAT6$_{(4-17)}$ I-A^b MHC II tetramer. The representative dot plots and bar chart above shows the relative frequencies of the activated ESAT6$_{(4-17)}$ I-A^b specific antigen CD4 (CD44$^+$ CD62L$^-$) T-cells. Results are shown as mean ± SD of 4 - 5 mice per group, (*P ≤ 0.01, **P ≤ 0.001; two-way ANOVA with Tukey's multiple comparison).

3.3.2.4 Dysregulated IL-27 signalling results in increased frequencies of MuFTs

The protective capability of T-cells during infection with intracellular pathogens is gauged on their ability to produce inflammatory cytokines and vaccine studies give an insight to what

CD4 T-cell subset confer immune protection against TB. A T-cell immune response that elicits the production of MuFTs co-expressing TNF, IFNγ and IL-2 positively correlates with protection from infection[178,182]. In this thesis, it was examined whether a loss in IL-27 signalling would improve induction of MuFT cells with age. Consequently, young and old C57BL/6 and IL-27Rα$^{-/-}$ mice were infected with 100 CFU of *Mtb* and at the indicated timepoints, single cell suspension from lung tissue was stimulated with anti-CD3/CD28, labelled for flow cytometry and the relative proportion of MuFT-cells was evaluated. At 21 days post infection, where the other 3 groups show that the frequencies of MuFTs are similar in the young cohorts and old C57BL/6 mice, the frequency of MuFT in older IL-27Rα$^{-/-}$ mice was already significantly increased. On day 43 post infection, frequency of MuFT cells in young and old C57BL/6 mice were lower than albeit without statistical significance that what was measured on day 21. In young IL-27Rα$^{-/-}$ mice the relative proportion of MuFT cells was only slightly increased. The infiltration with MuFT cells in old IL-27Rα$^{-/-}$ mice was similar on day 21 and day 43 (Fig. 35A)
Another potent proinflammatory cytokine produced by CD4 T-cells during primary *Mtb* infection is IL-17A. This cytokine indirectly facilitates the recruitment of protective cells and promotes granuloma formation in the lungs. As previously described, the production of IL-17A and the expansion of TH17-cells during experimental TB is limited by the suppressive activity of IL-27[178]. To date, it is not clear whether this is a direct effect of TH17-cells or an indirect effect of IL-27 (e.g. TR1 cells). To examine if the frequency of TH17-cells with increasing age is also affected by IL-27 signalling, C57BL/6 and IL-27Rα$^{-/-}$ young and old mice were infected with 100 CFU of *Mtb*. At the indicated timepoints post infection, single cell suspensions from lung tissue were prepared, labelled with antibodies for flow cytometry, and analysed. On day 21 post infection, no significant difference in the frequencies of TH17-cells was observed between young C57BL/6 and IL-27Rα$^{-/-}$ mice as was the case in old C57BL/6 and IL-27Rα$^{-/-}$ mice. On day 43 post infection, a similar trend was observed (Fig. 35B). This result contradicts previous findings where the loss of IL-27Rα resulted in increased numbers and frequencies of IL-17A-producing TH17-cells[178]. Taken together, the results tell of a role IL-27 might play in inducing the production of proinflammatory multifunctional CD4 T-cells during *Mtb* infection in older mice and although the results do not show increased IL-17A production, abrogated IL-27 signalling results in an increased in other inflammatory mediators.

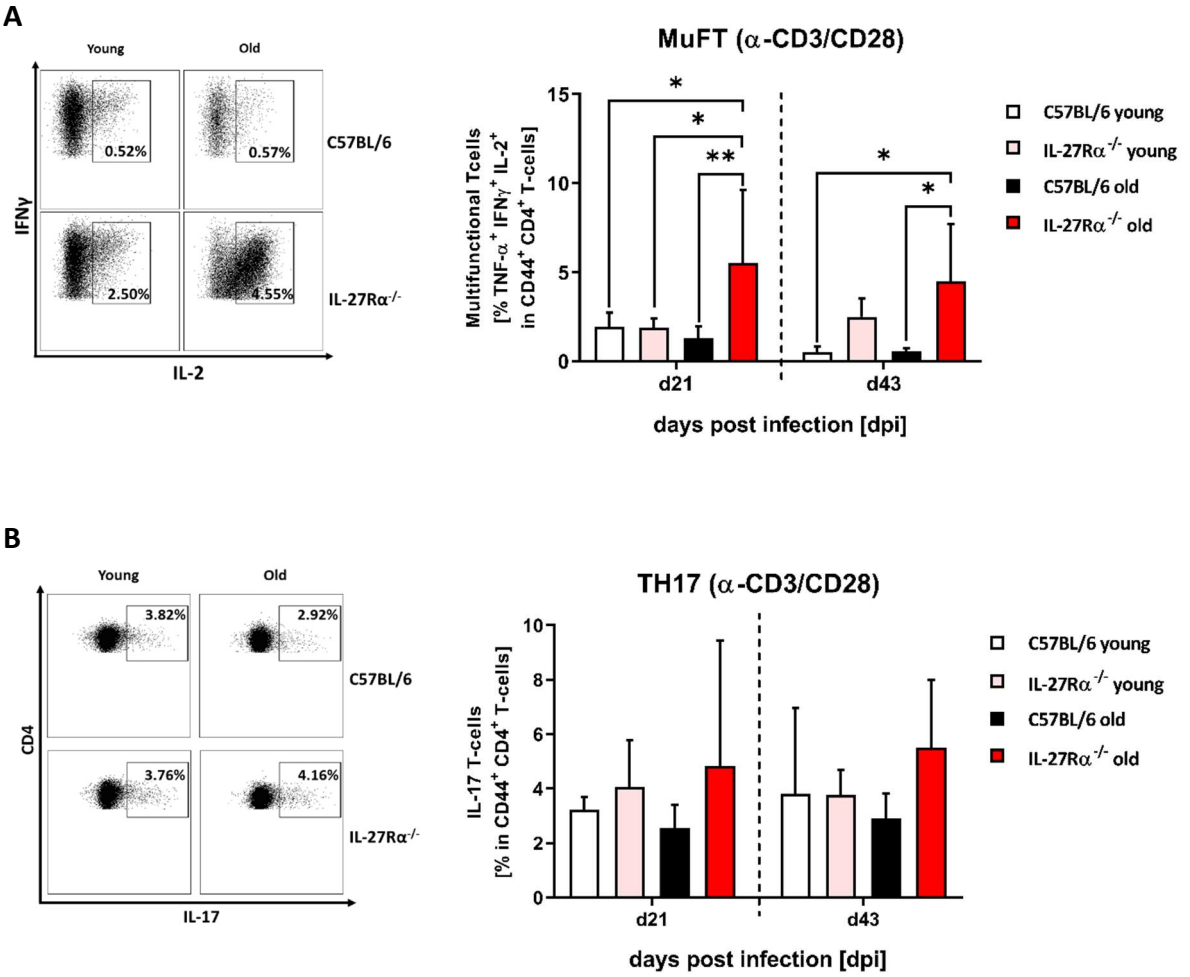

Figure 35. Increased frequencies of MuFT and TH17-cells in aged IL-27Rα$^{-/-}$ mice infected with *Mtb*
Young (4 mths old) and old (14 mths old) C57BL/6 and IL-27Rα$^{-/-}$ mice were infected via aerosol with 100 CFU *Mtb*. At the indicated time points after infection, single cells suspension from the lungs were obtained, stimulated for 5 h with anti-CD3/CD28 and labelled with Abs against CD90.2, CD4, CD44, IL-17, IL-2, TNF and IFNγ. The representative dot plots and bar charts above show the relative frequencies of MuFTs **(A)** and TH17 T-cells within the CD4$^+$ CD44$^+$ T-cells. Results are shown as mean ± SD of 4 - 5 mice per group, (*P ≤ 0.01, **P ≤ 0.001; two-way ANOVA with Tukey's multiple comparison).

In summary, the results in the present thesis show that although old mice have a higher frequency of MuFTs and TH17-cells during *Mtb* infection than young mice, their presence does not reduce the number of viable bacteria in the lungs of old mice. This lack of bacterial control is probably due to the age-related increase in TFH-10 and TR1 regulatory cells, which could interfere with effector T-cell functions. Indeed, when TR1 cells are significantly reduced in IL-27Rα$^{-/-}$ mice, *Mtb* growth is better controlled.

4 DISCUSSION

Extension of human lifespan is an incredible feat of modern medicine[1]. However, for most individuals, longevity does not automatically come with improved health quality but rather with increased risks for sickness, infections and impaired vaccine response caused by immunosenescence and inflammaging. With aging affecting both arms of the immune system, including reduced pathogen uptake, increased ROS and cytokine production by cells of the innate immune system, or alterations to CD4 and CD8 T-cell numbers, it is important to have a comprehensive understanding of the many facets of changes that occur in the immune system during aging.

The effects of aging on the immune system are particularly noticeable in CD4 T-cell populations because of the central role they play in inducing and mediating the adaptive immune response. The enhanced production of inflammatory cytokines by CD4 T-cells[64,183] alters their differentiation and subsequent immune responses. Indeed, genome studies on CD4 T-cells of old mice found distinct subsets with pro-inflammatory signatures that accumulated with age[184]. This age-associated increase in pro-inflammatory mediators was previously attributed to declining levels of the anti-inflammatory cytokine IL-10, but recent studies describe an increase in regulatory T-cell numbers and a resultant increase in IL-10 with age[90,121,123]. However, it is still unclear whether this increase in IL-10 originates from the $FoxP3^+$ or the $FoxP3^-$ regulatory T-cell subset. Consequently, the present thesis aims to characterised IL-10 production in both $FoxP3^+$ and $FoxP3^-$ populations of CD4 T-cells.

IL-6, a recognised marker for determining the severity of inflammaging[114], is responsible for the induction of $IL\text{-}10^+$ CD4 T-cells[24]. It presumably induces the degradation of FoxP3[185] and promotes the accumulation of $FoxP3^-$ regulatory cells[95]. However, the mechanism by which IL-6 signalling modulates of this $FoxP3^-$ regulatory population in aging is yet to be understood, as the complex nature of IL-6 signalling, involving at least three different modes, makes this challenging. Therefore, the work in the present thesis sought to understand how IL-6 signalling in old age contributes to increased $FoxP3^-$ $IL\text{-}10^+$ CD4 T-cells.

The concrete role $FoxP3^-$ $IL\text{-}10^+$ regulatory CD4 T-cells play in increasing susceptibility to infections in the elderly is yet to be elucidated. What previous research show is that regulatory cells increase at infection sites. For example, a study using mouse models of Leishmania infection showed an increased accumulation of $FoxP3^+$ and $FoxP3^-$ regulatory cells at the site

of infection. Both these cells produced IL-10, but FoxP3$^-$ CD4 T-cells were the predominate source of IL-10[186]. In another study of patients with active TB, an impaired MHC-II-mediated delayed hypersensitivity response (DTH) was associated with a poorer disease outcome due to high circulating IL-10 levels and impaired CD4 T-cell response to mycobacterial antigens. This increase in circulating IL-10 was revealed to come from TR1-like cells with potent antigen-unspecific immunosuppressive properties[187]. In *Mtb* infections, these FoxP3$^-$ regulatory cells have only been addressed in the relation to cutaneous anergy. However, in the context of pulmonary tuberculosis during aging, the effects of these FoxP3$^-$regulatory CD4 T-cells have yet to be investigated. If these FoxP3$^-$ CD4 T-cells, like FoxP3$^+$ CD4 Tregs, are increased with age[90], it is safe to assume that FoxP3$^-$ regulatory cells are also readily recruited to infection sites, suppress effector cells functions and allow for easy establishment of the infective organism. Therefore, the work in this thesis also aims to investigate the relationship between FoxP3$^-$ regulatory CD4 T-cells, aging and establishment of a lung infection using an experimental TB model.

The results of the present thesis show a direct correlation between IL-6 and the accrual of regulatory FoxP3$^-$ CD4 T-cells. Furthermore, it confirmed that the frequency of TR1 and TFH-10 cells increase with age and are sources of increased circulating IL-10. These cells are also increased in the lungs during *Mtb* infection in the elderly and can therefore be targeted for depletion to better modulate the immune response.

4.1 Aging is associated with increased IL-10 production from CD4 T-cells

IL-10, originally identified as a product of TH2-cells is also produced by myeloid cells, B-cells and different regulatory CD4 T-cells. Following stimulation, IL-10 blocks the activation or release of inflammatory mediators and therefore serves as an immune regulator. In recent years, interest in the involvement of IL-10 in immune aging and diseases development has increased. In studies on age-related diseases, IL-10 reportedly had protective properties. It inhibited endothelial dysfunction and inflammation in blood vessels by abating the effects of angiotensin II[188]. Increased IL-10 production in centenarians with the *IL10* promoter SNP 1082G→A was also associated with healthy aging[104]. Conversely, a deficiency in IL-10 is beneficial to hosts during infections as studies using *Leishmania* major[186] or *Mycobacterium* spp[189] showed reduction in bacterial burden in the absence of IL-10. These studies highlight the protective and damaging properties of increased IL-10 production on immune function.

Measurement of IL-10 in elderly individuals has engendered some conflicting results, with some reporting decreased IL-10 secretion in older individuals while others detected increasing IL-10 levels with age[121,122]. Whereas these studies measured IL-10 levels in elderly humans after stimulation or during infection, the work in the present thesis measured basal level of IL-10 in serum and found that it was increased in older mice. Although aging increased overall circulating IL-10 level, this increase was not reflected in every tissue or cellular compartment of older mice as qRT-PCR revealed that spleen, lymph nodes and white adipose tissue were the sources of increased IL-10 production in old mice. The increased IL-10 in lymphoid organs can directly limit the differentiation of effector T-cell and possible explains the poor control of infections, and dampened vaccine response in old age.

Conflicting results on changes in the innate immune system with age makes it difficult to define them as the main source of IL-10. Moreover, IL-10 production in young mice was found to be concentrated in B- and T-cells[157], suggesting that these cells are likely sources of IL-10 in advanced age. Indeed, previous studies described B-cells as the source of increased IL-10 production during aging. They showed that the treatment of aged mice with dehydroepiandrosterone sulphate (a natural steroid which declines with age) reduced B1 cell numbers and reversed excessive IL-10 production[190,191]. However, since these experiments were performed on B1 cells, which make up less than 5% of the total splenic B-cells, it follows that they cannot be responsible for the increased IL-10 production in old age. A later study examining total B cells refuted the claim that IL-10-producing B cells increase with age[192], thus suggesting other sources for the increased IL-10 production in old age. While the data generated in the present thesis could not conclusively exclude B-cells as the main IL-10-producing cells, they do suggest that CD4 T-cells are a likely IL-10 source during aging, and given that CD4 cells have high IL-10 reserves, as seen in infections where IL-10 production by these cells is markedly increased[193-196], they are ideal candidates for the cellular source of increased IL-10 production in old age. Notably, a study found increasing numbers of IL-10-producing $CD4^{+}CD44^{hi}$ T-cells with age[121].

4.2 Lineage specificity of $FoxP3^{-}$ $IL\text{-}10^{+}$ CD4 regulatory T-cells in aged mice

IL-10 production by CD4 cells was originally attributed only to TH2-cells, as it can be stably expressed by these cells after differentiation[197]. In more recent studies, IL-10 has also been described as a property of other TH-cell subpopulations such as TH1 or TH17[159,198]. However,

since these subpopulations are assumed to have no regulatory functions, as they do not respond anergically to TCR stimulation or suppress T-cell proliferation[199], it would be misleading to quantify IL-10 in all CD4 T-cells. Hence, the focus of the present thesis was on the regulatory CD4 T-cell populations

A source of indispensable immunoregulatory IL-10 are professional regulatory T-cells that are either Foxp3$^+$ or FoxP3$^-$ in nature. There is evidence linking the immunosenescent phenotype in advanced age to increased IL-10 secretion from FoxP3$^+$ CD4 T-cells[90], although our recent publication disputes this claim[158]. When we depleted FoxP3$^+$ Tregs in aged mice, it did not result in a decrease in serum IL-10 but rather in its increase[158]. This indicates that sources other than FoxP3$^+$ CD4 T-cells also contribute to increased IL-10 with age. Furthermore, preliminary results showed an increase in IL-10 production from FoxP3$^-$ CD4 T-cells after depletion of Foxp3$^+$ Tregs[200] [Unpublished doctoral dissertation] suggesting them as a likely source of increased IL-10 in advanced age. Results in the present thesis revealed an association between aging and increased FoxP3$^-$ CD4 T-cells. It also identifies these cells as a source of elevated IL-10 with age.

These FoxP3$^-$ IL-10$^+$ CD4 T-cells were thought to be derived from functional and genetic reprogramming of other TH-cell types. For example, Gagliani et al., demonstrated a trans-differentiation of TH17-cells to TR1-like cells that expressed IL-10 (exTH17 ^{TR1}) and were distinct from the original TH17 population in young mice[159]. The authors suggested these trans-differentiated cells are exTH17^{TR1} cells, but the findings in the present thesis do not corroborate this claim. Since TH17-cells transiently express IL-10 and markers associated with TR1 cells such as CD49b or the cytosolic transcription factor aryl hydrocarbon receptor (AhR)[96,201], it is possible that the cells determined to be exTH17^{TR1} cells had temporarily downregulated typical TH17 markers in favour of TR1 markers. It is also conceivable that a small fraction of TR1 cells is derived from TH17-cells, but these have no significance in the context of aging. Beyond trans-differentiation, transient or complete loss of FoxP3 in old Tregs was thought to lead to the development of FoxP3$^-$ IL-10$^+$ CD4 T-cells[202] However, for Tregs to maintain suppressive or regulatory activities, continuous expression of FoxP3 is a requirement[202]. Furthermore, when Tregs lose their FoxP3 expression and become exTregs, they contribute to the aging phenotype by increasing inflammation, as they are themselves capable of producing proinflammatory cytokines such as IL-17A or IFNγ and can drive autoimmunity[202,203] including type 1 diabetes or experimental autoimmune encephalomyelitis

(EAE). But regardless of their pro-inflammatory signature, exTregs are still capable of producing IL-10. Results in the present thesis revealed similar frequencies of IL-10-producing exTregs in both young and old mice. Despite similar exTreg frequencies, young mice did not have the typical features related to immune aging such as an overall increased basal IL-10 level. Therefore, IL-10-producing exTregs can be excluded as the source of increased IL-10 and as mediators of immunosenescence/inflammaging in old age. Instead, the results of the present thesis imply that FoxP3$^-$ IL-10 regulatory cells are their own distinct population. Indeed, this thesis identified two separate FoxP3$^-$ cell populations that express TFH (called TFH-10) and TR1 markers as potential sources for the age-related increase of IL-10. One study in mice, for example, found that continuous exposure to LCMV resulted in an increase in IL-21 and IL-10 secreted by CD4 T-cells, which increased the TFH-10 population[204]. This chronic exposure to viral antigens and the subsequent functional adaptation of CD4 T-cells in mice might reflect the human situation, in which an aged immune system with long term exposure to CMV or other antigen stimulants, does not only lead to clonal expansion of activated T-cells, but also to an increase in TFH-10 cells. Similarly, persistent antigenic stimulation is required for the activation of transcription factors (*Irf1* and *Batf*) that are involved in TR1 differentiation in mice[205]. Therefore, antigenic insults over the course of a lifetime results in increased frequencies of TFH-10 and TR1 cells.

4.3 IL-6: master regulator of FoxP3$^-$ IL-10$^+$ CD4 T-cell with age

Although progress is being made in understanding different subsets of CD4 T-cells, there is no clear insight to the exact mechanisms underlying the accrual of the Foxp3$^-$ regulatory subsets with age. It is known that the cytokine milieu in which CD4 TH-cells are stimulated dictates their polarisation. Research shows that advancing age, progressively skews the immune response towards a pro-inflammatory phenotype that results in a low-grade chronic inflammation and the subsequent degradation of FoxP3[206,207]. Whereas others report this inverse association between pro-inflammatory cytokines like IL-6 and FoxP3 expression on regulatory CD4 T-cells, the result in the present thesis shows that deficiencies in IL-6 has little to no effect on FoxP3 expression (Fig. S4). Rather, there is a significant correlation between increased IL-6 in old age and the accrual of a FoxP3$^-$ IL-10$^+$ cell population that is comprised mainly of TFH-10 and TR1 cells. This ability of cells to secrete both pro- and anti-inflammatory cytokines in parallel, is an age-old story, where it is often observed that an increase in

pro-inflammatory mediators inadvertently leads to an increase compensatory anti-inflammatory mediators such as IL-10. The exact mechanism by which IL-6 increases this FoxP3$^-$ population with age is still unclear, but IL-6 stimulation of naïve CD4 T-cells in the presence of STAT3 inhibitors results in a marked reduction of IL-10 production *in vitro*[95], suggesting the involvement of STAT3 in the accrual of this regulatory population.

4.3.1 No direct impact of IL-6 classical or trans-signalling on TFH-10 or TR1 cell accrual

The pleiotropic nature of IL-6 allows it to possess both pro- and anti-inflammatory characteristics. Compared to its inflammatory effects, mechanisms of anti-inflammation by IL-6 are not well understood. In some inflammatory diseases, IL-6 plays an indispensable role in suppressing T-cell infiltration by regulating IL-10 production and it is thought to enact this regulatory function via classical signalling and activation of JAK/STAT proteins[208].

Initial results in the present thesis suggested that IL-6 signalling via the classical signalling route would result in an increase in TFH-10 and/or TR1 cells. However, the absence of enhanced STAT1 Tyr701 or STAT3 Tyr705 phosphorylation following induction of the classical signalling pathway by IL-6 stimulation in older mice, indicates that this signalling route has no effect on the accrual of TFH-10 or TR1 cells with age. Consistent with this observation, older mice were found to have less of the membrane-bound IL-6Rα that enables classical signalling in cells. Additionally, the inducible knockdown of the membrane-bound IL-6Rα did not affect TFH-10 or TR1 populations, confirming that classical signalling is not involved in the maintenance of the FoxP3$^-$ regulatory CD4 T-cells. However, it is possible that the classical IL-6 signalling pathway is necessary for the initial development of FoxP3$^-$ regulatory CD4 T-cells, which then accumulate with time, such that a transient knockdown of the classical signalling pathway in old mice has no effect on the frequencies of already established TFH-10 or TR1 populations.

The alternative trans-signalling route for IL-6, involving the binding of IL-6 to its soluble receptor and gp130, is described as a potent activator of inflammatory responses[127,209]. Because increased IL-6 with age is associated with inflammatory diseases and frailty, the circulating IL-6 likely signals via trans-signalling and inadvertently increases IL-10-producing cells. Furthermore, inflammation significantly impairs the expression of membrane-bound IL-6Rα and increases the serum concentration of sIL-6Rα at infection and inflammatory sites[209]. The increase in sIL-6Rα and the decreased expression of membrane-bound IL-6Rα

could explain why inflammatory diseases are often diagnosed in the elderly, as a reduction of membrane-bound IL-6Rα on CD4 T-cells of old mice, would force the cells to signal via the alternative trans-signalling route, thereby increasing inflammation. In addition, activation of trans-signalling is also determined by the ratio of IL-6Rα to gp130 available on the cell surfaces. One study found that when gp130 expression was higher than membrane-bound IL-6Rα, cells were forced to signal via trans-signalling[210]. In addition to the increased levels of sIL-6Rα, the results in the present thesis also reveal that the frequency of gp130 expression is higher than IL-6Rα on CD4 T-cells of older mice and therefore suggests an increased use of the trans-signalling pathway with age. Although the results in the present thesis indicate that aging results in increased activation of IL-6 trans-signalling, enhanced activity of this pathway could be excluded since the stimulation of CD4 spleen cells with HyIL-6 did not result in increased STAT phosphorylation and neither were TFH-10 nor TR1 cell populations reduced in old mice deficient in IL-6 trans-signalling.

Trans-signalling-induced responses are regulated by the presence of its natural antagonist, sgp130, that ameliorates inflammation in animal models[211,212]. The present thesis demonstrates an age-related increase in sgp130 and therefore, suggests a reduced capacity for IL-6 trans-signalling in old mice. Remarkably, the regulatory functions of sgp130 extend beyond simply blocking trans-signalling. At high concentrations, sgp130 was able to block IL-6 classical signalling by forcing free IL-6 into an IL-6/IL-6Rα/sgp130 complex[213]. Thus, in older mice, not only is trans-signalling inhibited, but there is also an indirect blockage of classical signalling mediated by sgp130. One can theorise that this regulation of IL-6 signalling and the subsequent attenuation of inflammatory responses is important for healthy aging and the reversal or slowing of inflammaging processes and immunosenescence.

In addition to phosphorylation on its tyrosine residue, phosphorylation on the serine 727 residue occurs as a secondary event for maximal activation of STAT3 [166], and although research on the phosphorylation of STAT3 at the Ser727 residue has increased in recent years, particularly in the fields of cancer and cardiology, its role from an immunological perspective has taken a back seat. However, there is evidence that phosphorylation of STAT3 at its serine residue contributes to the maintenance of IL-21 production by CD4 cells in the presence of IL-6 by regulating Ca^{2+} in mitochondria[214]. It is therefore possible that STAT3 Ser727 activation in involved in increasing TFH-10 cells in old age.

STAT3 Ser727 phosphorylation is mediated by the activation of different kinases including the mechanistic target of rapamycin (mTOR) which is involved in many age-related defects, as inhibition of the TORC1 or TORC2 complex likely attenuates the aging phenotype by reducing Tregs, improving CD4 TCR signalling in a TORC2 complex dependent manner[169], restraining the conversion of memory T-cells to $CD28^-$ TEM cells and diminishing IL-10 secretion[215]. Although its role in TFH differentiation is controversially discussed, mTOR has been shown to govern TFH cell differentiation via its TORC complexes. Whereas one study showed that inhibition of mTORC1 by rapamycin or small hairpin RNA (shRNA) promoted TFH differentiation during LCMV infection[216], other studies found that loss of mTOR signalling reduced TFH differentiation and GC responses in mice[217,218], suggesting that inhibiting mTOR enhances effector cell response while also regulating IL-10 accumulation. However, as none of these experiments were conducted in the context of aging and regulatory cell function, the precise effect of mTOR on TFH-10 cells with age remains elusive.

Given the lack of information on the effects of mTOR signalling on TR1 cells, but by virtue of its involvement in regulating numerous TH-cell differentiations, a reasonable assumption is that mTOR signalling also affects the abundance of TR1 cells during aging.

While the results in the present thesis do not shed light on how mTOR is affected by aging and if indeed it alters TFH-10 and TR1 frequencies, the result shows that phosphorylation of STAT3 on its Ser727 residue increases with age. With mTOR being one of the kinases that phosphorylates STAT3 at Ser727, it can be suggested that mTOR activity also increases during aging.

STAT3 activation is associated with the increase in IL-10[118]. However, the present thesis revealed that a transient knockdown of STAT3 on CD4 T-cells did not result in changes in the frequencies of TFH-10 or TR1 cells in old mice. This result does not necessarily rule out STAT3 activity in the accrual of TFH-10 or TR1 cells. It could simply mean that the duration of the knockdown was not sufficiently long enough to cause any change to TFH-10 and TR1 populations. Secondly, the abundance of various STAT proteins might lead to certain redundancies, so that a knockdown of STAT3 might activate compensatory mechanisms from other STAT proteins.

The results in the present thesis so far suggest that phosphorylation of tyrosine residues by IL-6 classical or trans-signalling is dispensable for TFH-10 or TR1 accrual with age. Rather,

activation of the STAT3 protein by phosphorylation on its serine residue might play a more important role.

4.3.2 IL-6 governs TFH-10 and TR1 accrual with age by regulating IL-21 and IL-27 secretion by T-cells

The present thesis sheds new light on the role of IL-6 during aging. Despite a general increase in pro-inflammatory mediators in the aging immune system, a decline in immune response to new antigens or vaccination is observed in elderly individuals[3]. One explanation for this is that IL-6 regulates the secretion various cytokines and the expression of genes related to the differentiation of FoxP3$^-$ regulatory CD4 T-cell subsets (Fig. 36). IL-6 commits cells to a TFH lineage by driving IL-21 production and increasing Bcl-6 mRNA expression[139]. It also triggers the secretion of IL-27, a TR1 inducing cytokine[140,219]. Therefore, increased circulating IL-6 suggest that cytokines downstream of IL-6 are also increased. Indeed, serum levels for IL-21 and IL-27 were elevated in old mice which is in accordance with human studies that report elevated levels of IL-21 and IL-27 in sera of elderly individuals[154,220]. Furthermore, the knockout of IL-6 resulted in a reduced secretion of both IL-21 and IL-27 in CD4 T-cells.

The increase of IL-21 and IL-27 were previously associated to age-associated inflammation. However, the work in the present thesis directly links IL-21 and IL-27 to the accrual of IL-10-producing TFH cells (TFH-10) and TR1 cells with age. In other studies, the expansion of these FoxP3$^-$ regulatory cells is described as an indirect effect of IL-27 that is mediated by IL-21[161,221], and therefore define IL-21 as a downstream target of IL-27. Consistently, results in this thesis show that the loss of IL-21 significantly reduces both populations of TFH-10 and TR1 cells in old mice. Unlike the previous studies that describe an association between IL-27 and TFH differentiation[221], abrogation of IL-27 signalling in the present thesis only resulted in a significant reduction of TR1 cells but not TFH-10 cells in old mice. One explanation is that IL-27 signalling also determines TH-cell lineage at the molecular level. For example, IL-27 signalling promotes the expression of the transcription factor Blimp-1, which then inhibits TFH differentiation by blocking Bcl-6 transcription[222].

Amongst the series of transcription factors involved in the development of TFH cells, cMAF is strongly induced in TFH differentiation and its expression is associated with increased production of IL-21 and IL-10[161,223,224]. IL-21 likely acts in an autocrine fashion to transactivate cMAF, which in turn leads to a further activation of IL-21 genes. In accordance, results from old IL-21$^{-/-}$ mice show decreased cMAF expression in TFH-10 and TR1 cells. Additionally,

TFH-10 and TR1 cells of IL-6$^{-/-}$ mice also show a decrease in cMAF expression. This is because IL-6 indirectly activates cMAF via the IL-21/STAT3 pathway[139,225]. Another underlying mechanism for cMAF expression and IL-10 production is the IL 27-dependent STAT3 signalling and activation of downstream elements[161]. Furthermore, IL-27 induces the transcription of AhR that in combination with cMAF optimises the transactivation of *IL21* and *IL10* promoters[226]. In line with this, cMAF expression in TFH-10 cells of old IL-27Rα$^{-/-}$ mice is significantly reduced whereas TR1 cells only show a slight reduction in cMAF expression. This suggest that IL-27 via the activation of cMAF can trigger IL-10 production in TFH-10 cells of old mice but might employ a different strategy when stimulating IL-10 production from TR1 cells. EGR2 expression characterises cells as anergic and these anergic cells share similarities with regulatory cells[227]. In old age, suboptimal TCR stimulation results in long-term hypo-responsiveness and a subsequent increase in EGR2. This weak TCR stimulation is essential for IL-27 mediated EGR2 induction[219]. EGR2 promotes IL-10 production by activating Blimp-1 in an IL-27/STAT3 dependent pathway[219] and confers T-cells with LAG3^{+} phenotype (Fig. 36), a typical marker for TR1 and immune exhaustion[219,228,229]. LAG3 in conjunction with IL-10 contributes to the regulatory nature of TR1 cells. Indeed, the result in the present thesis revealed that older mice express significantly more ERG2 than younger mice. Unlike in TFH-10 cells, EGR2 was significantly reduced in TR1 cells of old IL-27Rα$^{-/-}$ mice and suggests the importance of that TR1 differentiation via EGR2 and IL-27 signalling. In addition to IL-27, IL-6 induces EGR2, Blimp-1 and IL-10 expression in a STAT3 dependent manner[219] which is consistent with the results of the present thesis showing a significant reduction of EGR2 and IL-10 in old IL-6$^{-/-}$ mice. The marked decrease of EGR2 in the absence of IL-6, IL-21 or IL-27 signalling elucidates the importance of these cytokines in the development and maintenance of TFH-10 and TR1 cells.

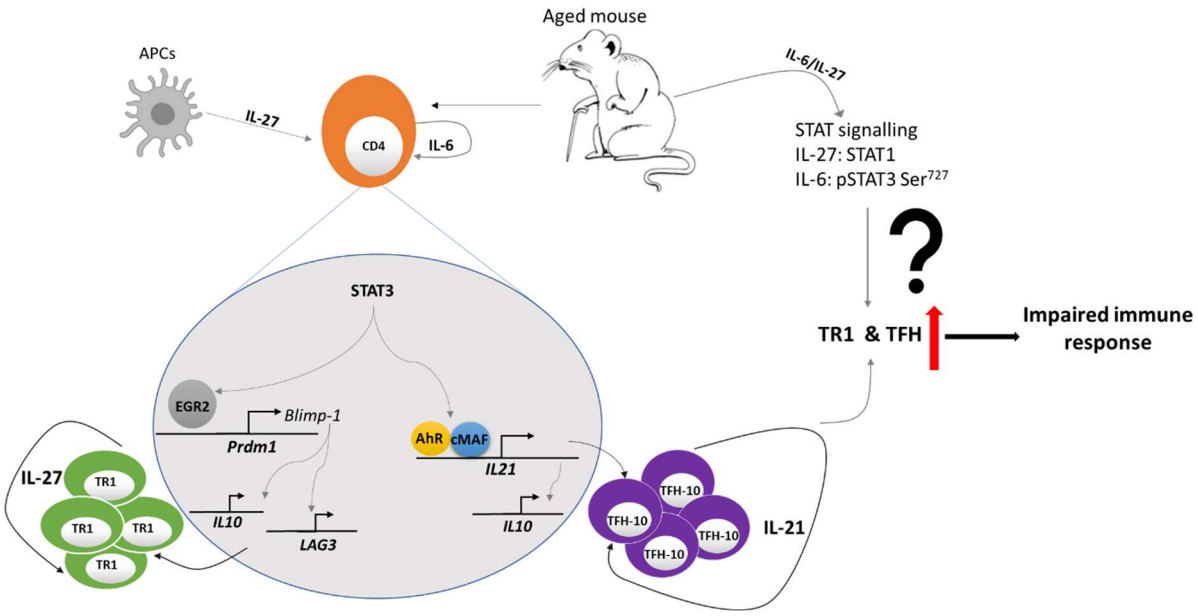

Figure 36. Cytokine induced generation of TFH-10 and TR1 cells
Increased IL-6 and IL-27 production with age activates STAT3 dependent signalling pathways that promote the expression of the transcription factor EGR2 and induction of Blimp-*1* which is critical for the activation of LAG3 and IL-10 and a consequent increase in LAG3^{+} IL-10 TR1 cells. STAT3 dependent IL-27 signalling induces AhR and cMAF that synergistically enhance IL-21 and IL-10 production from TFH-10 cells. IL-21 and IL-27 produced by TFH-10 and TR1 respectively, can act in an autocrine manner to further enhance their generation. IL-10 produced by TFH-10 and TR1 cells suppress effector T-cell and inhibit optimal immune responses.

Although STAT3 inducing cytokines are associated with transcription factors that trigger IL-10 production in CD4 T-cells, the experiments carried out in the present thesis could not verify the precise mechanisms of the accrual of TFH-10 and TR1 cells by STAT3 activation. This therefore needs further examination.

4.3.3 Antigen-dependent and independent action of TFH-10 and TR1 cells

Aged individuals and mice have increases levels of IL-10 production from various regulatory CD4 T-cell subsets[90,121,158]. Indirectly, IL-10 can inhibit mediators of the adaptive immune system by forcing them to express tolerogenic molecules which leads to the induction of even more IL-10-producing regulatory cells[99]. IL-10 also acts directly on T-cells in a suppressive manner either by inhibiting cytokine expression or halting proliferation and the differentiation of effector cells subsets[119].

Within the compartment of regulatory CD4 T-cells, TR1 cells have been reported to exhibit high regulatory activity, mainly due to the action of IL-10[97,230]. However, two things stood out in these studies. 1). All FoxP3^{-} IL-10^{+} CD4 T-cells were always classified as TR1 cells and not further subdivided into other populations. 2). Antigen-specific TR1 cells were used when investigating the suppressive capacity of TR1 cells. In the present thesis, FoxP3^{-} IL-10^{+} CD4

T-cells were divided into two distinct populations and the antigen-specific and -unspecific suppressive activity of both populations was tested individually. TR1 cells were found to suppress Teff proliferation only slightly in an antigen-independent manner. A possible explanation is that TR1 cells need to be antigen-specific to effectively suppress Teff cell proliferation[230], which could explain why significant inhibition of CD4 Teff cell proliferation was not achieved. The present thesis also failed to demonstrate an antigen-specific suppressive effect for TR1 cells. One reason for this could be that a high number of TR1 cells is required to suppress Teff cell proliferation *in vitro*. Indeed, others show an antigen-dependent suppression of proliferating cells by TR1 cells only when the ratio of TR1 cells is much higher than that of effector cells[230]. This therefore suggests that an abundance of TR1 cells in required for effects of the suppression of this population to be measurable *in vitro*.

For a long time, TR1 cells were classified as the main suppressive cells of the FoxP3$^-$ CD4 T-cell population. However, the result in the present thesis has expanded on this population and describes TFH-10 as a second subset of FoxP3$^-$ cells that also secret high levels of IL-10. This finding is consistent with a study in children in which a population of TFH cells lacking FoxP3 expression, with high IL-10 secretion and the ability to supress T-cells proliferation was also found in the tonsils[231]. These tonsillar TFH cells suppressed immune responses and controlled inflammation to foreign antigens in an antigen-independent manner. Consistent with the dominant role of IL-10 in immune suppression, the results in the present found that the suppression of Teff proliferation mediated by TFH-10 is antigen-dependent as well as -independent and relies on IL-10, because a blockade of the IL-10R abolished the suppressive effect of TFH-10 cells.

The result in the present thesis unveils TFH-10 cells as more potent suppressor cells than the previously described TR1 cells and suggest them as likely targets for modulation to counter the effects of immunosuppression in old age.

4.4 Implication of *Mtb* infection in aged hosts

Although *Mtb* infection occurs in all age groups, post-primary TB most often occurs in elderly individuals. Using a mouse model, the work in the present thesis tries to delineate how TFH-10 and TR1 cells populate the infected lung in *Mtb* infected young and old mice and how a modulation of these cells affects the disease progression in aged animals.

The outcome of *Mtb* infection depends on a well-orchestrated immune response that encompasses both the innate and adaptive immune system. Because the cells of the innate immune system are relatively intact during aging, the increased susceptibility to TB with age is probably a result of defects in the adaptive immune responses. Besides, others have shown that control of *Mtb* infection is dependent on a CD4 T-cell TH1 response with the production of cytokines such as IFNγ, IL-12 and TNF[232]. This therefore indicates that the decline in CD4 TH1 response with age contributes to the increased susceptibility of elderly individuals to *Mtb* infections. Indeed, initial susceptibility studies demonstrated an inability of old mice to control *Mtb* growth within lungs or peripheral organs after an intravenous infection[23]. This inability to control infection was a result of the defective accumulation of CD4 T-cells. In contrast, a low dose aerosol infection of old mice showed a transient control of bacterial growth at the early stages of infection that is associated with increased numbers of circulating CD8 T-cells[232]. These CD8 T-cells accumulate over a lifetime due to previous pathogen exposure, are pre-activated and can therefore participate in the immune response to *Mtb* in an antigen-independent manner[233,234]. This activation of CD8 T-cells likely occurs via NK-associated receptors that are increasingly expressed on aged CD8 T-cells[235]. The present thesis confirms the ability of older mice to control bacterial growth only transiently. Whereas others showed old mice could control bacterial growth similarly to young mice up to 90 days post infection[232], the present thesis revealed that younger mice had a significant reduction in bacterial load as early as 72 days post infection compared to older mice.

Young mice effectively control bacterial growth early in infection, but unlike old mice, they do so by recruiting antigen-specific CD4 T-cells to their lungs, and this recruitment of antigen-specific CD4 T-cells in young mice is noticeable as early as 3 weeks post infection [233]. Interestingly, results in the present thesis reveal that old mice elicit a similar immune response as young mice when *Mtb* infection progresses beyond 21 days. The frequency of infiltrating CD4 T-cells to the infected lungs of young and old mice was not significantly different (result not shown). Furthermore, the frequency and number of CD4 T-cells positive for ESAT-6 antigen were comparable between young and old mice. It is possible that the cells generated in younger mice are more effective in controlling the bacteria because inflammaging profile in older mice results in the constant stimulation of immune cells, and as research has shown, persistent stimulation of CD4 T-cells causes cell exhaustion, terminally differentiation and a failure to confer protection against infections[176]. Alternatively, the delayed response of

antigen-specific CD4 T-cells by older mice enables replication and growth of bacteria to numbers that are far too great to be effectively controlled when antigen-specific cells are eventually recruited. Further experiments to test the efficacy and the kinetics of the antigen-specific cells produced in old mice could help clarify why the bacteria load was not reduced at the measured time points when compared to young mice.

Overall, the results presented here indicate that old mice can elicit an antigen-specific immune response when infected with *Mtb*. Despite the similarities in immune response between young and old mice, older mice still showed increased bacterial burden. Another conceivable explanation would be production of IL-10 from regulatory T-cells at infection sites. These cells are recruited at the same rate as effector cells to the site of infection during *Mtb* infection[174]. In pro-inflammatory settings, these regulatory cells are beneficial to the host, as they would minimise the occurrence of TB lesions by dampening inflammatory responses. However, their immunosuppressive properties are also beneficial to *Mtb* growth. Given that the frequency of regulatory cells increases with age, their abundance could promote the growth and spread of bacteria.

4.4.1 Cytokines of effector and regulatory CD4 T-cell immune response in *Mtb* infection during aging

IL-6 is upregulated and plays a dual role during *Mtb* infections, as mice deficient in IL-6 were found to have a reduced TH1 response and increased bacterial loads after high doses of intravenous *Mtb* infection[236]. However, the effects of IL-6 where dispensable for the generation of specific immunity or control of aerosol *Mtb* infection[237-239]. In human studies, increased IL-6 expression is associated with a more rapid bacterial clearance[240]. While these studies may suggest that old individuals benefit from increased inflammatory mediators due to inflammaging, the long-term consequences of IL-6-induced inflammation likely affect *Mtb* infection inflammatory responses and granuloma formation. In mice, for example, IL-6 drives the differentiation of TH17-cells that are responsible for enhanced protection against *Mtb* and granuloma formation. However, an excessive induction of TH17 response during *Mtb* infection in mice drives neutrophilic pathology and *Mtb* growth[241]. Indeed, the results in this thesis show an increased TH17 response in old C57BL/6 mice, although they still have higher bacterial loads than younger mice. The increased production of IL-17A may also result in increased production of IL-10 from lung regulatory cells such as TR1, which act as a control for TH17 responses. Indeed, human studies show an association between increased IL-17 and

IL-10 in latent TB patients who later converted to active TB cases[242]. Along with enhancing inflammatory mediators, IL-6 secretion has also been shown to have detrimental effects on *Mtb* immune responses. In macrophages, it caused a reduction in IFNγ-induced uptake of *Mtb* and a downregulation of the effective targeting of the TORC1 complex of mTOR by IFNγ, resulting in increased IL-10 production[243]. Furthermore, the results in the present thesis and work carried out by others[95] highlight the crucial role of IL-6 in the accrual of regulatory TFH-10 and TR1 cells with age. These TR1 cells are recruited to the lung during *Mtb* infection[178] and secrete high amounts of IL-10 that could possibly suppress the immune response to the infection.

IL-10 is often used as a correlate for TB susceptibility and limits the immune system's control of *Mtb* infection[244,245]. Consistent with its role in TB pathogenesis, IL-10 deficiency in the relatively resistant C57BL/6 mice resulted in a subtle reduction in *Mtb* bacterial load and an increase in IFNγ production[245]. This relationship between IL-10 and increased susceptibility was better demonstrated in experiments using susceptible CBA/J mice, which normally have higher basal levels of IL-10. Blockade of IL-10 signalling in these CBA/J mice resulted in reduced bacterial burden that was associated with an enhanced TH1-cell response[246]. Although results in the present thesis revealed that IL-10 is increased in older mice, this increase had no effect on multifunctional CD4 TH1-cell (IFNγ/TNF/IL-2) response. Admittedly, older mice had higher frequencies of multifunctional CD4 TH1-cells during *Mtb* infection, yet the magnitude of this immune response was not sufficient to effect bacterial clearance. It is possible that the excessive or prolonged activation of these multifunctional CD4 T-cells causes them to become exhausted and less effective[247].

There are varying reports on the cellular source of increased IL-10 production during *Mtb* infection. In one study, the macrophages of transgenic mice that overexpressed IL-10 were found to have a marked decrease in bactericidal function resulting in increased bacterial burden, although TH1-cell differentiation and function was unaffected[125]. However, in another study, IL-10 production by CD4 T-cells is described as the main culprit for an increased susceptibility to *Mtb* infections, because deletion of IL-10 specifically in CD4 T-cells made mice more resistant to *Mtb* infections than that their wild-type cohorts[248]. Findings in the present thesis show that under sterile conditions CD4 T-cells in the elderly produce the most IL-10 within the adaptive immune system. This therefore suggests that an infection with *Mtb* is more easily established in the elderly, as IL-10 production from CD4 T-cells is an important

determinant in *Mtb* infection establishment. The results from human studies also parallel those of mouse studies, with IL-10 as well as TGF-β being elevated in bronchoalveolar lavage (BAL) fluid and serum from patients with active pulmonary tuberculosis[244]. Given that TGF-β is required for TR1 differentiation[201], the increase in TGF-β during *Mtb* infection might further increase TR1 cells in the lungs of aged individuals. Indeed, Gerosa et al., identified a population of TR1-like *Mtb* antigen-specific CD4 T-cells that secreted both IL-10 and IFNγ in BAL fluid that was ineffective in reducing bacterial burden[249]. In the present thesis, the population of TR1 and TFH-10 cells are found to be increased in the lungs of old infected mice. Collectively, these studies suggest that excessive IL-10 production impairs control of *Mtb* infection. With age, frequencies of regulatory TFH-10 and TR1 cells increases and may provide the necessary IL-10 needed to suppress immune surveillance and lead to new infections or post-primary TB in the elderly.

In the present thesis, an IL-6 dependent increase in the frequency of TFH-10 and TR1 cells was first detected in aged mice. Since infection with *Mtb* expands and activates regulatory T-cells in the lungs, the work here sought to determine if an infection with *Mtb* would have the same effect on TFH-10 and TR1 cells during *Mtb* infection in old mice. Similar to reports finding an increase in infiltrating Tregs[173,250], the present thesis is also the first to report an increased frequency of TFH-10 and TR1 cells in the infected lungs of old mice. This increase in TFH-10 and TR1 cells is induced by *Mtb*, because splenic TFH-10 and TR1 cells frequency were also increased in infected old mice. TR1 cells are thought to protect tissue damage under normal conditions by blocking TNF production and limit TH17 response[251]. In the case of *Mtb* infection, blocking the effects of TNF or TH17-cells is detrimental and increases susceptibility to TB disease. It is therefore plausible that an increase in TR1 plays a role in increasing susceptibility to severe outcomes of TB disease in the elderly.

It is at this point not clear the exact role TFH-10 cells play in the pathogenesis of TB during aging. One likely effect of increased TFH-10 cells in the elderly is that it will aid in the production of antibodies that opsonise *Mtb*, thereby making them more permissive for uptake and growth within macrophages. Another outcome is that these TFH-10 cell, similar to Tregs and TR1 cells, also act in a regulatory manner by secreting high levels of IL-10 and suppressing the immune response to *Mtb* infection.

4.4.2 High-grade inflammation inhibits pathogen clearance

IL-27, a cytokine with anti-inflammatory properties on a broad range of TH immune responses, is elevated during active TB in humans and hinders an effective immune response[252]. Indeed, studies in young mice highlight the immunoregulatory role of IL-27, because deletion of IL-27Rα resulted not only in enhanced bacterial clearance but also in increased immunopathology[178,180].

The results in the present thesis demonstrated that loss of IL-27 signalling reduces the population of TR1 cells in old mice. Therefore, the disruption of IL-27 signalling the subsequent reduced frequencies of TR1 cells should have increased protective immune responses in old mice. Indeed, the frequencies of ESAT-6 specific and multifunctional CD4 T-cells were increased in old IL-27Rα$^{-/-}$ mice after *Mtb* infection, but even this increase in TB-related protective immune response in old IL-27Rα$^{-/-}$ mice did not lead to a better control of bacterial growth, but rather to an increase in bacterial load. This finding suggests that inflammation has a bidirectional effect on infection, with the moderate inflammatory milieu in young IL-27Rα$^{-/-}$ mice providing an environment that is detrimental to bacterial growth, whereas the excessive inflammatory state in old IL-27Rα$^{-/-}$ mice, exacerbated by aging, promotes bacterial growth. Indeed, this hyper-inflamed milieu promoting bacterial growth, has been previously reported in other diseases[253]. With regards to TB, Brace et al., show that, inhibition of PI3K/Akt/mTORC1 metabolism increases expression and secretion of the MMP-1 gene and several proinflammatory cytokines[254]. This increase in inflammatory mediators enables TB pathology, increase in bacterial load and transmission of *Mtb* bacteria. In co-infection with HIV and *Mtb*, Interferon-gamma release assay positive (IGRA^{+}) patients with an increased frequency of IFNγ^{+} IL-17^{+} cells progressed to active TB, while IGRA^{+} patients with more IL-10^{+} IL-17^{+} cells had a more specific TH17 response that was protective[242]. In the present study, the pre-existing proinflammatory state in older mice may create an enabling environment that promotes bacterial growth.

The results presented here support the concept that a tightly regulated inflammatory and anti-inflammatory response is required for optimal immune protection. Although IL-10 produced by TR1 and TFH-10 cells can be detrimental during infections, the loss of these cell populations and their control over the inflammatory response could lead to unsuccessful aging as well as impaired bacterial clearance in infections such as tuberculosis.

4.5 Why healthy aging matters

Aging is a risk factor for developing chronic diseases and increased susceptibility to infections. Therefore, the shift in age demographic will have a profound impact on health systems worldwide. Aging is a multifactorial process making it is difficult to specify exact mechanisms that determine if an individual would have a healthy lifespan. Currently, the medical strategies that cater to this growing aging population is largely based on treating of symptoms of diseases as they arise.

Studies using various species have vastly improved our understanding on aging processes and demonstrate that targeting one or more hallmarks of aging can delay onset of aging and age-related diseases. Of particular interest is inflammaging and the accompanying increase in circulating inflammatory mediators such as IL-6, that contributes to the immunosenescent phenotype in the elderly. Inflammaging reflects not only an increase in proinflammatory markers but also a concomitant increase in anti-inflammatory mediators. Indeed, results presented in this thesis show that the loss of IL-6 signalling reduces the number of IL-10-producing TFH-10 and TR1 regulatory cells, suggesting that blocking IL-6 may improve the immune response in the elderly. However, treatment of rheumatoid arthritis patients with tocilizumab, an IL-6R blocker, is associated with increased risk of infections[255]. Based on the results of the present thesis, it also appears that depletion of TR1 cells does not improve control of *Mtb* infection in old mice, even though proinflammatory mediators increase after TR1 depletion. Overall, these data suggest that a balance between pro- and anti-inflammatory mediators dictate the difference between immunopathological and healthy immune responses. This hypothesis is reflected in human studies in Italian centenarians, where increased IL-6 and a simultaneous increase in IL-10 production is associated with longevity[103,104]. Conversely, when IL-10 production is much higher than inflammatory mediators, a negative effect on the immune response to infection and vaccination is expected[115].

With a rapidly aging society, the cost of care and treatment for the elderly will rise, making preventive medical procedures such as immune modulation a viable strategy to mitigate the adverse effects of inflammaging in our aging population, but such intervention must achieve a fine-tuned balance between the secretion of pro- and anti-inflammatory mediators. Achieving this could lead to a lower incidence of age-related diseases and a better vaccine response in the elderly.

Summary

Recent theories on aging immune systems describe a dysregulation in the cytokine network, with a predisposition towards increased pro-inflammatory cytokine production, called "inflammaging". Paradoxically, aging is also accompanied by the increase in immune modulatory mediators, characterised by increase in IL-10. Both inflammaging and anti-inflammaging have profound effects on the immune response to pathogens. IL-6 is used as a measure of inflammaging and is reported to induce IL-10 production from T-helper cell subsets. In this thesis, the effect of IL-6 signalling on the accrual of FoxP3$^-$ IL-10$^+$ CD4 T-cells and their impact on infection was explored and the following results were obtained:

- A link between increased IL-6 and increased regulatory T-cells that lack FoxP3 expression was established. These FoxP3$^-$ IL-10$^+$ CD4 T-cells are predominantly TFH-10 and TR1 cells.
- Neither IL-6 classical- nor trans-signalling is involved in the accrual of TFH-10 or TR1 cells.
- Loss of IL-6 in aged mice alters the expression of transcription factors like cMAF and EGR2. These transcription factors are implicated in the accrual of TFH-10 and TR1 cells.
- While TFH-10 cells are able to suppress T-cell proliferation in an antigen-independent manner, TR1 cells do not possess this trait.
- Although the inflammatory response in older mice during *Mtb* infection is higher than in younger mice, older mice could not effectively control the growth of *Mtb*.
- Increasing the inflammatory state by elimination of the TR1 population in older mice does not improve *Mtb* clearance.

The work in this thesis demonstrates the importance of a balance in pro-inflammatory and anti-inflammatory immune mediators for healthy aging. Based on these results, IL-10-producing cells dampen the immune responses to infections or vaccinations but also protect the host from exacerbated immune responses.

Zusammenfassung

Jüngste Theorien über das alternde Immunsystem beschreiben eine Dysregulation im Zytokinnetzwerk mit einer Tendenz zur vermehrten Produktion von entzündungsfördernden Zytokinen, die als "Inflammaging" bezeichnet wird. Paradoxerweise geht die Alterung auch mit einem Anstieg der immunmodulierenden Mediatoren einher, der durch einen Anstieg von IL-10 gekennzeichnet ist. Sowohl Inflammaging als auch Anti-Inflammaging haben tiefgreifende Auswirkungen auf die Immunreaktion auf Krankheitserreger. IL-6 wird als Maß für die Entzündung verwendet und induziert Berichten zufolge die IL-10-Produktion von T-Helferzellen-Untergruppen. In der vorliegenden Arbeit wurde die Auswirkung der IL-6-Signalisierung auf die Bildung von FoxP3$^-$ IL 10$^+$ CD4 T-Zellen und ihre Auswirkungen auf die Infektion untersucht:

- Es wurde ein Zusammenhang zwischen erhöhtem IL-6 und vermehrten regulatorischen T-Zellen hergestellt, denen die FoxP3-Expression fehlt. Bei diesen FoxP3$^-$ IL-10$^+$ CD4 T-Zellen handelt es sich überwiegend um TFH-10- und TR1-Zellen.
- Weder die klassische noch die trans-Signalisierung von IL 6 ist an der Entstehung von TFH-10- oder TR1-Zellen beteiligt.
- Der Verlust von IL-6 bei alten Mäusen verändert die Expression von Transkriptionsfaktoren wie cMAF und EGR2. Diese Transkriptionsfaktoren sind an der Vermehrung von TFH-10- und TR1-Zellen beteiligt.
- Während TFH-10-Zellen in der Lage sind, die Proliferation von T-Zellen antigenunabhängig zu unterdrücken, verfügen TR1-Zellen nicht über diese Eigenschaft.
- Obwohl die Entzündungsreaktion bei älteren Mäusen während einer *Mtb*-Infektion stärker ist als bei jüngeren Mäusen, konnten ältere Mäuse das Wachstum von *Mtb* nicht wirksam kontrollieren.
- Die Erhöhung des Entzündungszustandes durch Eliminierung der TR1-Population in älteren Mäusen verbessert die *Mtb*-Clearance nicht.

Die vorliegende Arbeit demonstriert, wie wichtig ein Gleichgewicht zwischen entzündungsfördernden und entzündungshemmenden Immunmediatoren für ein gesundes Altern ist. Basierend auf diesen Ergebnissen dämpfen IL-10 produzierende Zellen die Immunreaktionen auf Infektionen oder Impfungen, schützen aber auch den Wirt vor überschießenden Immunreaktionen.

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Appendix

Supplementary Figure 1

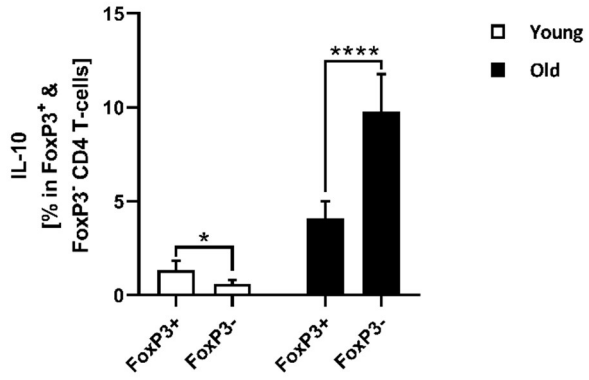

Figure S1. Increased IL-10 production from FoxP3-negative CD4 T-cells in old mice
After a 5 h stimulation with PMA/Ionomycin and Brefeldin block, single cell suspension of spleen cells from C57BL/6 young (2 mths old) and old (17 mths old) mice were fluorescent labelled with Abs against TCR-β, CD8, FoxP3 and IL-10. Using Flow cytometry, the relative percentage of IL-10 in $CD4^+$ $FoxP3^+$ and $CD4^+$ $FoxP3^-$ populations were determined. (*P ≤ 0.04, **** P ≤ 0.0001; Two-way ANOVA with Sidak's multiple comparison test)

Supplementary Figure 2

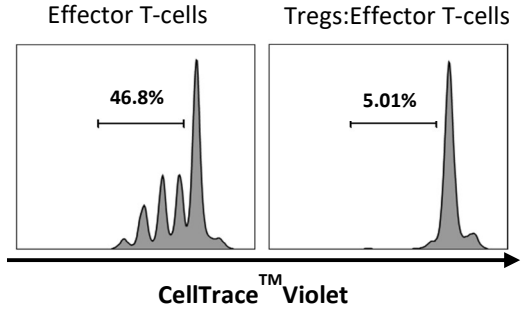

Figure S2. FoxP3-positive regulatory T-cells inhibit cell proliferation
Spleen cells form $FoxP3^{DTR}$ mice were enriched for CD4 and sorted on GFP into FoxP3-positive Treg cells. Effector T-cells were cultured with or without Tregs for 3 days. Result shows the dilution of the proliferation dye and the inhibition of proliferation of effector cells by Tregs

Supplementary Figure 3

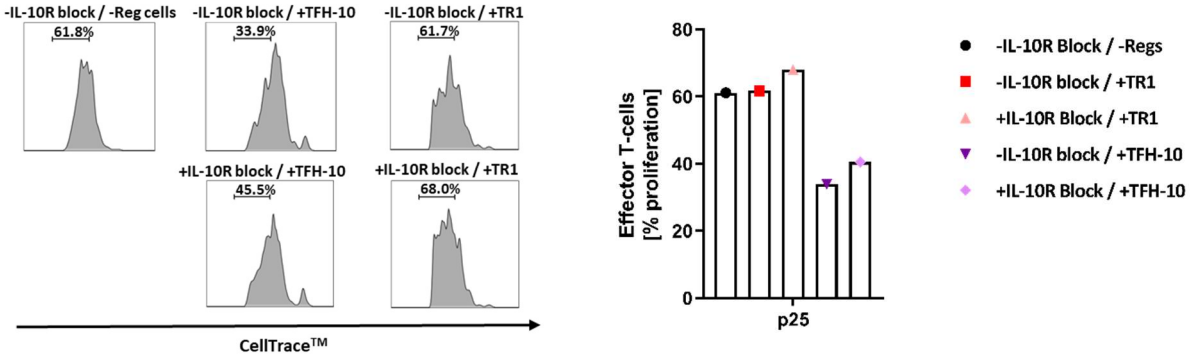

Figure S3. Antigen specific suppression stronger with TFH-10 cells
Spleen cells form old p25 mice were enriched for CD4 and sorted into Lag3-negative effector T-cells or FoxP3-negative TFH-10 and TR1 cells. Effector T-cells were cultured with or TFH-10 (1:3) or Tr1 cells (1:5) for 3 days. Result shows the dilution of the proliferation dye and the inhibition of proliferation of effector cells by regulatory CD4 T-cells

Supplementary Figure 4

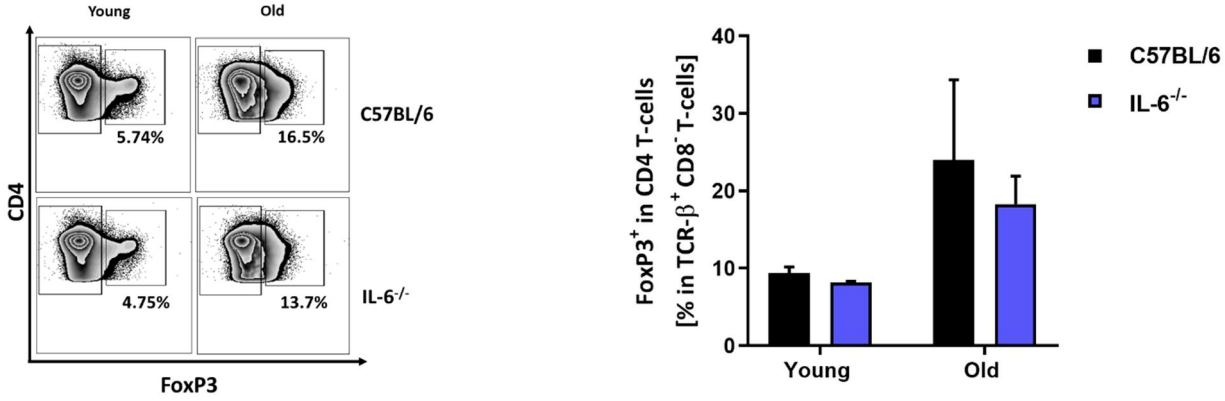

Figure S4. IL-6 deficiency does not significantly impair FoxP3 expression in old mice
After a 5 h stimulation with PMA/Ionomycin and Brefeldin block, single cell suspension of spleen cells from C57BL/6 young (2 mths old) and old (17 mths old) mice were fluorescent labelled with Abs against TCR-β, CD8, FoxP3 and IL-10. Using Flow cytometry, the relative percentage of $FoxP3^+$ in $CD4^+$ population was determined

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Publications

Journal Papers

Regulation of the Mitochondrion-Fatty Acid Axis for the Metabolic Reprogramming of *Chlamydia trachomatis* during Treatment with β-Lactam Antimicrobials. *mBio* **12** (2021).
Kensuke Shima, Inga Kaufhold, Thomas Eder, Nadja Käding, Nis Schmidt, **Iretiolu M. Ogunsulire**, René Deenen, Karl Köhrer, Dirk Friedrich, Sophie E. Isay, Florian Grebien, Matthias Klinger, Barbara C. Richer, Ulrich L. Günther, George S. Deepe Jr., Thomas Rattei, Jan Rupp

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Conferences

2017 Minisymposium“ Infektion und Immunabwehr“, Rothenfels, Deutschland **Talk**	***Ogunsulire I.,*** *Varela F., Behrends J., Agger E. M., Almanan M., Hildeman D. and Hölscher C*. Vaccination efficacy against tuberculosis is enhanced by regulatory T cell depletion.
2017 Immunology Symposium, Deer Creek, Columbus, Ohio **Poster**	***Ogunsulire I.,*** *Almanan M., Hildeman D. and Hölscher C.* Characterisation of FoxP3^{-} IL-10^{+} CD4^{+} T-cells in young and aged mice
2018 5th European congress of immunology (ECI), Amsterdam, Nederland **Talk**	***Ogunsulire I.,*** *Almanan M., Hildeman D. and Hölscher C.* Characterisation of IL-10-producing CD4 T cells in aged mice
2019 Allergy meets inflammation (AMI) Lübeck, Germany **Talk and Poster**	***Ogunsulire I.,*** *Almanan M., Hildeman D. and Hölscher C.* Changes in cytokine signalling influences the expression of FoxP3-negative regulatory T-cells with age
2019 New developments in immunology (NDI$_3$) Borstel, Germany **Poster**	***Ogunsulire I.,*** *Almanan M., Hildeman D. and Hölscher C.* Cytokines the promote the accrual of FoxP3^{-} IL-10^{+} CD4 T cells in aged mice