Deciphering the *in vivo* mechanisms underlying the IL-10-mediated differentiation of IFNγ-producing T cells.

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Abstract

Interleukin (IL)-10 is a potent anti-inflammatory cytokine that plays a central role in the development of an appropriate immune response. IL-10 is particularly known for inhibiting cytokine production and antigen presentation by activated myeloid cells. Due to its role in limiting and terminating inflammatory responses, the therapeutic potential of IL-10 has been considered for chronic inflammatory conditions. Although low concentrations of IL-10 are well tolerated and present an immunosuppressive capacity, both patients and healthy volunteers receiving therapeutic doses of IL-10 developed marked side effects such as headaches, fatigue, anemia, neutrophilia, monocytosis and thrombocytopenia.

Emergency myelopoiesis (EM) is a hematopoietic response triggered by stress cues that skews hematopoiesis towards the production of myeloid cells. Neutrophilia, monocytosis and thrombocytopenia are characteristic of EM. In many models, the production of pro-inflammatory cytokines, such as interferon γ (IFNγ), drive EM. However, whether anti-inflammatory molecules may play a role in this process remains elusive.

Recent work from our group found that *in vivo* exposure to IL-10 induced the differentiation of IFNγ-producing CD4, CD8 and TCRγδ T cells. In turn, IFNγ initiates a global transcription program on the bone marrow hematopoietic precursors that culminates in EM. Altogether, these observations suggest that IL-10 may in fact play a dual role, having simultaneous anti- and pro-inflammatory activity. These opposing, context-specific, actions of IL-10 raise important issues related to its use in the clinic and call for further research into the pleiotropic nature of this cytokine.

The aim of my thesis was to unveil the cellular and molecular pathways that mediate the IL-10 reprogramming of CD4 and CD8 T cells towards an inflammatory state. We demonstrated that IL-10 reprograms T cells into a cytotoxic state, leading to cell dysfunction which seems to trigger cellular senescence. We further show an impact of IL-10 in adipose tissue senescence, likely mediated by dysfunctional T cells. Understanding the impact and mechanisms of action of IL-10 will open new perspectives for the design of IL-10 based immunotherapies.

Keywords: IL-10, T cells, IFNγ.

Resumo

A interleucina (IL)-10 é uma citocina anti-inflamatória muito potente com um papel central no desenvolvimento de respostas imunes apropriadas. IL-10 é particularmente conhecida pela sua capacidade de inibir a produção de citocinas e apresentação de antigénios por parte de células mieloides ativadas. Devido a esta sua capacidade em limitar e terminar respostas inflamatórias, IL-10 foi sugerida como uma possível via de tratamento para doenças crónicas inflamatórias. Apesar de a baixas concentrações a IL-10 ser bem tolerada e apresentar propriedades imunossupressoras, ambos pacientes e voluntários saudáveis que receberam doses terapêuticas de IL-10 desenvolveram efeitos secundários tais como dores de cabeça, fatiga, anemia, neutrófila, monocitose e trombocitopenia.

Mielopoiese de emergência (EM) é uma resposta hematopoiética provocada por sinais que desviam a hematopoiese para a produção de células mieloides. Em vários modelos, a EM envolve a produção de citocinas pró-inflamatórias tais como o interferão γ (IFNγ). Investigações recentes do nosso grupo revelaram que a exposição a IL-10 *in vivo* leva á diferenciação de células T CD4, CD8 e TCRγδ produtoras de IFNγ. Por sua vez, o IFNγ dá início a um programa transcripcional global nos precursores hematopoiéticos da medula óssea, culminando no desenvolvimento de EM. Todas estas observações sugerem que a IL-10 poderá desempenhar uma dupla função, tendo simultaneamente propriedades pró e anti-inflamatórias. Estes efeitos opostos e dependentes do contexto levantam dúvidas importantes relativas ao uso clínico da IL-10, sendo necessário mais estudos sobre as propriedades pleiotrópicas desta citocina.

O objetivo da minha tese foi revelar os mecanismos moleculares e celulares que medeiam a reprogramação das células T CD4 e CD8 para um estado inflamatório. Demonstramos que a IL-10 reprograma as células T para um estado citotóxico, levando ao desenvolvimento de disfunções celulares o que parece desencadear senescência nas células. Ainda demonstramos um impacto da IL-10 na senescência do tecido adiposo, provavelmente mediado por células T disfuncionais. Um melhor conhecimento desde mecanismo irá abrir novas perspetivas para o desenvolvimento de imunoterapias envolvendo a IL-10.

Palavras-Chave: IL-10, células T, IFNγ.

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List of abbreviations

ATP- Adenosine triphosphate

BM – Bone marrow

CCR7 - C-C chemokine receptor type 7

CLP – Common lymphoid progenitors

CMP – Common myeloid progenitor

COVID-19 - Coronavirus disease 2019

CTLA-4 – Cytotoxic T-lymphocyte antigen 4

CTLs – Effector cytotoxic T lymphocytes

DCs – Dentritic cells

DN – Double negative

DNA-SCARS - DNA segments with chromatin alterations reinforcing senescence

DP – Double positive

ECT – Electron Transport Chain

EM - Emergency myelopoiesis

FBS – Fetal bovine serum

FOXP3 – Transcription factor forkhead box P3

Glut1 – Glucose transporter 1

GMP – Granulocyte/macrophage progenitor

HSC - Hematopoietic stem cells

H&E - Hematoxylin and eosin

IBD - Inflammatory bowel disease

IFNγ – Interferon γ

IL-10 – Interleukin-10

IL-10R – Interleukin-10 receptor

KLRG-1 – Killer Cell lectin-like receptor subfamily G member

LAG-3 – lymphocyte activated gene-3

LSK - Lineage$^-$ Sca-1$^+$ c-Kit$^+$ cells

MTG – MitoTracker green

MTR – MitoTracker red

NAD^+ - Nicotinamide adenine dinucleotide

NK – Natural Killer

OXPHOS – Oxidative phosphorylation

PBS – Phosphate-buffered saline

PD-1 – Programmed cell death 1

rIL-10 – Recombinant IL-10

RNA-seq – RNA sequencing

ROS – Reactive Oxygen Species

SASP- Senescence-associated secretory phenotype

SA-β-gal – Senescence-associated β-galactosidase

SIRT-1 – Sirtuin 1

STAT3 – Signal transducer and activator of transcription 3

T_{CM} – Central memory T cells

TCR – T cell receptor receptor

T_{EM} – Effector memory T cells

T_{fh} – Follicular helper T cells

TGFβ – Transforming growth factor β

Th – T helper

TIM-3 – Mucin domain-containing protein 3

TNF-α – Tumor necrosis factor-α

T_{reg} - Regulatory T cell

Zn - Zinc

yH2AX – Phosphorylation of histone H2AX

Introduction

Interleukin-10

Brief introduction to interleukin-10

Interleukin (IL)-10 is an important anti-inflammatory cytokine, responsible for hindering the development of exuberant inflammatory responses and a key player in several other immunologic contexts, such as wound healing, cancer and autoimmunity [1]. This cytokine is produced by various subsets of CD8 T cells and CD4 T cells (including T helper (Th)1, Th2 and Th17 cell subsets), regulatory T cells (T_{reg}) and B cells [2, 3]. Myeloid cells such as macrophages, monocytes, neutrophils, natural killer (NK), eosinophils, mast and dendritic cells (DCs) are also able to respond to stimulus by secreting IL-10 [2, 4, 5]. Interestingly, some nonimmune cells have also been shown to secrete IL-10, which is the case for fibroblasts and epithelial cells [6, 7]. However, IL-10 regulation is very complex and different cells produce IL-10 in different quantitates depending on the nature and strength of the stimuli [2].

After its production, in order to elicit a response, IL-10 will interact with cells bearing the IL-10 receptor (IL-10R) complex which is composed of two subunits, IL-10Rα and IL-10Rβ. Cells able to respond to IL-10 include both innate and adaptive immune cells, such as NK, DCs, monocytes, macrophages, neutrophils, B cells and CD4 and CD8 T cells [8]. Macrophages have been suggested to be the main target of IL-10 effects [9, 10], and this idea is supported by the fact that macrophages express high levels of IL-10R, when compared to other cell types [4]. IL-10 triggers, through its receptor, the activation of signal transducer and activator of transcription 3 (STAT3) [4]. The main mechanism for IL-10 functions is thought to be through the inhibition of transcription [11]. IL-10 is particularly known for suppressing inflammatory responses of innate immune cells, inhibiting cytokine production and antigen presentation by activated myeloid cells [9, 12-14]. It controls Th1 cell responses by constraining T cell activation and inhibiting pro-inflammatory responses in tissues [14] and modulates the suppression of proinflammatory IL-17 producing T cells (Th17) [15]. In addition, it plays a role in the survival and homeostasis of regulatory T cells [16, 17].

Interleukin-10 in disease and its therapeutic potential

In line with the immune suppressive effects of IL-10, disruption of the IL-10/IL-10R axis in both mice and humans is associated with exacerbated inflammation and underlies several diseases [10, 18, 19]. In this context, the development of inflammatory bowel

disease (IBD) has been associated with IL-10 receptor deficiencies, IL-10 polymorphisms and a dysfunctional IL-10 response in humans [19-21].

Given its anti-inflammatory potential, IL-10 has been proposed as a possible treatment for chronic inflammatory conditions [1]. The administration of recombinant IL-10 has been tested as a treatment for IBD, however the patients did not present a significant improvement [22-25]. IL-10 has been also tested in trials for other pathologies such as rheumatoid and psoriasis, where some promising responses were seen [26-28]. Overall, clinical trials testing IL-10 therapies demonstrated that low concentrations of IL-10 are well tolerated [22] and present an immunosuppressive capacity [29]. However, both patients and healthy volunteers receiving higher doses of IL-10 developed marked side effects such as headaches, fatigue, anemia, neutrophilia, monocytosis and thrombocytopenia, which are hematologic changes also seen in emergency myelopoiesis (EM) [30-34]. The mechanisms leading to this response are not yet clear. Previous studies have found that the administration of high doses of IL-10 enhanced interferon γ (IFNγ) production, limiting its anti-inflammatory activities and possible leading to the proinflammatory effects observed [35, 36].

IL-10 has also been widely studied in cancer, being the general view that the presence of high levels of IL-10 in the tumor-microenvironment typically correlates with poor prognosis [37, 38]. In contrast with this view, it has also been shown that the administration of a pegylated form of IL-10 induced the proliferation and activation of tumor-specific CD8 T cells, favoring effective T cell memory responses and increasing the production of both IFNγ and cytotoxic enzymes [15, 36, 39].

Non-classical roles of IL-10

In this controversial context regarding IL-10 and making use of the pMT-10 mouse model [40], our recent work contributes to the understanding of the mechanisms responsible for the hematologic alterations observed in human volunteers receiving therapeutic doses of recombinant IL-10 (rIL10). The pMT-10 mouse is an animal model of inducible IL-10 over-expression. The expression of IL-10 is controlled by a Zinc (Zn)- inducible sheep metallothionein promoter, resulting in increased IL-10 expression after Zn administration [40]. This model is ideal to study the impact of high doses of IL-10 to the homeostasis of the immune system.

We showed that IL-10 exposure *in vivo* acts as a trigger of EM, leading to alterations similar to those reported in humans undergoing IL-10 treatments [41]. High levels of IL-10 lead to an increase in lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cell and granulocyte/macrophage

progenitors (GMP) cell populations both in the spleen and bone marrow (BM) and promotes the differentiation of myeloid cells. We have furthermore shown that IL-10 reprograms the transcriptome of BM CD4 and CD8 T cells, towards the production of high levels of IFNγ. In turn, IFNγ initiates a global transcription program on the BM hematopoietic precursors that culminates in EM (Fig.1). This finding is supported by previous studies where IFNγ production in the BM was associated with over proliferation of hematopoietic stem cells (HSC) [42, 43] and in the context of bacterial infections, its production directly controlled the production of myeloid cells and skewed HSC towards the myeloid lineage [44, 45].

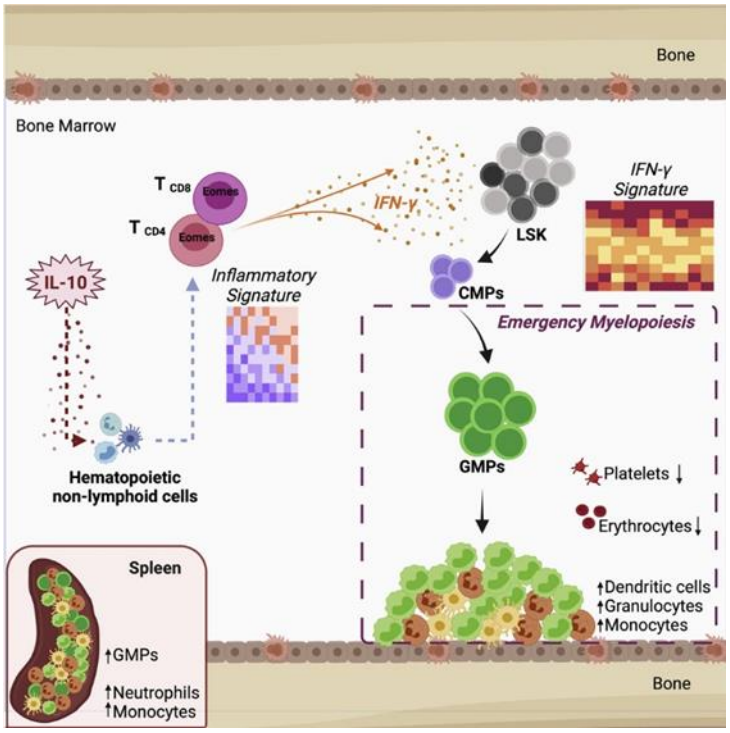


Figure 1: Schematic figure of the phenotype developed by mice after exposure to IL-10 *in vivo.* IL-10 over-expression led to a reprogramming of CD4 and CD8 T cells in the bone marrow (BM), characterized by a strong inflammatory signature and the production of high levels of IFNγ. IL-10 triggered the expansion of lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells and common myeloid progenitors (CMPs), whose differentiation is skewed into granulocytes-monocyte progenitor (GMPs), resulting in the development of emergency myelopoiesis (EM). Consequently, animals accumulate myeloid cells such as granulocytes, monocytes and dendritic cells and have a decreased in the number of platelets and hemoglobin in the blood. In the spleen there is also the expansion of GMPs accompanied by an accumulation of neutrophils and monocytes. Chimeric mice, pMT-10.IL10Rα$^{-/-}$ mice reconstituted with Ragγc$^{-/-}$ mice, retained an IL-10Rα deficient population of T cells, in the BM and spleen. IL-10 induction led to EM in these chimeras, suggesting that there are indirect mechanisms involved in IL-10 signaling in this setting, which does not exclude the possibility of a direct mechanism being also present. Image from *Cardoso et al* [41].

In accordance with our results other studies have also reported IL-10 functions that deviate from its traditional anti-inflammatory role. In the context of the coronavirus disease 2019 (COVID-19), patients that were treated with rIL10 and patients with severe COVID-19 presented the same cytokine expression profile, which included high levels of IL-4, IL-7, IL-18, IL-2Rα, IFNγ and tumor necrosis factor-α (TNF-α) [46-51]. High levels

of IL-10 were shown to provoke an increase of IFNγ-producing CD4 and CD8 T cells [52]. Furthermore, there is a correlation between the increase of exhausted programmed cell death 1 $(PD\text{-}1)^{+}$ mucin domain-containing protein 3 $(TIM3)^{+}CD8^{+}$ T cells in the peripheral blood of COVID-19 patients and the concentration of IL-10 in the serum, which indicates that IL-10 may play a role in T cell exhaustion [53].

Another non-classical role of this cytokine is its ability to limit thermogenic gene expression in adipose tissue, being able to work as a mechanism to conserve energy in a situation of infection. IL-10Rα is enriched in adipocytes and its expression is increased in cases of obesity and aging, which can indicate the presence of an unknown role of IL-10 in these contexts [54, 55].

It should also be mentioned that IL-10 is also increased in the serum of aged individuals [56], which once again reinforces IL-10 double role, since aging is highly associated with an increase in inflammation [57].

These studies, together with the hematologic alterations observed in individuals receiving IL-10 and the success of IL-10 therapies in pre-clinical models of solid tumors [58, 59], suggest that this cytokine may play a dual role, with simultaneous anti- and pro-inflammatory activity. These opposing actions of IL-10 raise doubts related to its use in the clinic, and therefore further research is necessary to understand the pleiotropic nature of this cytokine. Several open questions remain following our previously published work: How is IL-10 inducing T cell reprogramming in vivo? What functional alterations may occur in IL-10-differentiated T cells? Answering these questions is the aim of my thesis. In this context, the next section is focused on T cells.

The role and regulation of T lymphocytes

General introduction to T lymphocytes

The immune system is classically divided into two main subsystems, innate and adaptative, which are distinguished mainly by their degree of specificity and recognition repertoire [60]. The hallmark of the adaptative immunity is its capacity to maintain a memory of the antigens that it has been exposed to, allowing a more rapid reaction in case of a second encounter [61]. T lymphocytes are an essential part of this system [61, 62]. T cells derive from BM progenitors that migrate to the thymus. When in the thymus, common lymphoid progenitors (CLP) develop into $CD4^-CD8^-$ double negative (DN) T cells, later to $CD4^+CD8^+$ double positive (DP) T cells and finally into $CD8^+$ and $CD4^+$ single positive T cells (Fig.2) [63, 64]. These now mature T cells, exit the thymus and give origin to a pool of long-lived cells that populate the peripheral lymphoid tissues, circulating in a continuous manner between blood and lymph [65, 66]. Expression of CD4 or CD8, which are co-receptors taking part in the T cell receptor (TCR)-signal transduction, define two different T cell lineages: CD4 and CD8 T cells [67]. CD4 T cells are referred to as helper T cells, and their function is to promote or attenuate immune responses. CD8 are classified as cytotoxic T cells, as they have a direct protection role, due to their ability to kill cells expressing their target antigen [67, 68].

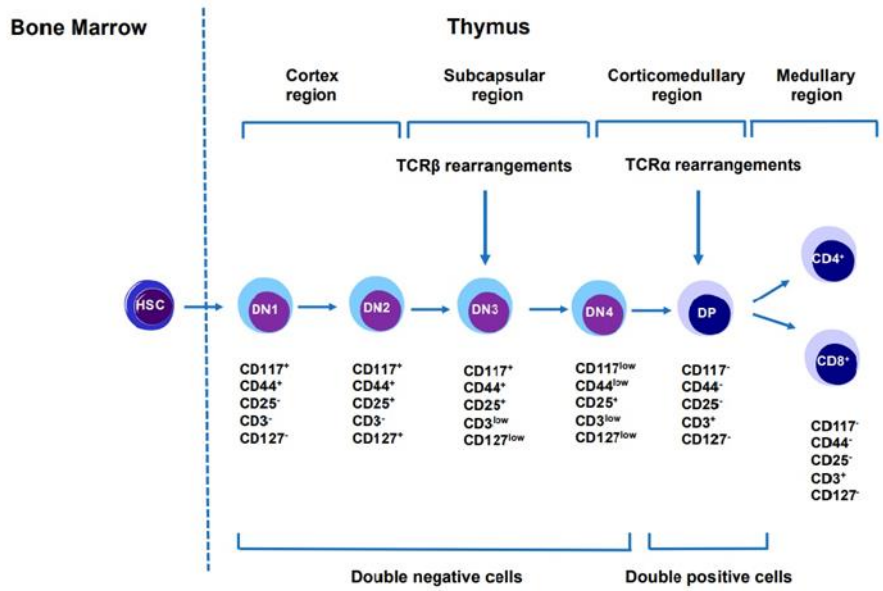


Figure 2: T cell development and maturation process. T cells derive from hematopoietic stem cells (HSC) that migrate into the thymus where T cell maturation occurs. Progenitors develop first into $CD4^-CD8^-$ double negative (DN) T cells, later to $CD4^+CD8^+$ double positive (DP) T cells and finally into $CD8^+$ and $CD4^+$ single positive T cells. Thymocytes in different stages of maturations can be identified through specific markers. Image from *Srivastava et al* [69].

T cell activation and differentiation

Both mature CD4 and CD8 cells can remain as naïve cells, meaning in a resting phase, for many weeks or months, rarely dividing [65]. Naïve cells are associated with low expression levels of the surface molecule CD44 and high levels of CD62L and C-C chemokine receptor type 7 (CCR7) [70]. The maintenance of the pool of naïve T cells is a complex process, that prevents cell activation by several mechanism such as the downregulation of genes involved in cell cycle progression [70, 71].

In a situation of exposure to antigens, naïve T cells quickly respond. This response can be separated into three main stages, expansion, contraction, and memory [72, 73]. During an immune response, naïve T cells become activated, clonally expand, and differentiate into effector T cells [65, 73], which present a high expression of CD44 [65]. These activated T cells are a short-lived population, where the majority of cells dies shortly after the immune response terminates, a process referred to as contraction. However, a small number of cells survives, originating a pool of antigen-specific memory T cells [66]. CD4 T cells can differentiate into different subsets upon TCR stimulation and under specific cytokine stimulation: Th1, Th2, Th9, Th17, Th22, T_{reg} and T_{fh} (follicular helper T cells), each subset producing hallmark cytokines [74, 75]. CD8 T cells differentiate into effector cytotoxic T lymphocytes (CTLs), acquiring the capacity to produce cytokines such as IFNγ and TNF-α [76].

Memory T cells are maintained for a long time, due to stimulation by molecules such as interleukin (IL)-7 and IL-15 [77, 78]. Differentiation of naïve T cells into memory cells is accompanied by a reprogramming of the transcriptome and the expression of genes that encode molecules such as IFNγ, perforin and granzyme B, which are not expressed in naïve cells [73]. Memory T cells can be found in secondary lymphoid organs, central memory cells (T_{CM}), or infected tissues, effector memory cells (T_{EM}). T_{CM} cells are characterized by high levels of CD62L expression, while T_{EM} have low expression of CD62L [79].

In a case of chronic inflammatory signals, due to persistent antigen stimulation or surrounding nutrient-poor microenvironment, such as a tumor environment, T cells can enter a state designated as exhaustion, which is characterized by the progressive loss of function, impaired cytotoxicity and low proliferative capacity [78, 80-83]. Although most studies have focused on CD8 exhausted T cells, both CD4 and C8 T cells are liable to developing an exhaustion phenotype [81, 84]. One hallmark of exhausted T cells is the higher expression levels of inhibitory receptors, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), TIM-3, lymphocyte activated gene-3 (LAG-3) and PD-1 [81, 85]. PD-1 is the

most studied checkpoint protein and thought to play a major role in exhaustion [78, 82]. PD-1 is upregulated in exhausted cells, when compared with effector T cells, where it is only transiently expressed upon activation [81].

Mature T cells retain a low degree of self-reactivity, that while still avoiding autoimmunity, enhances TCR sensitivity to foreign antigens [86]. This tolerance to tissue-specific antigens is further strengthened by T_{reg}, which display a strong immunosuppressive potential [87-90]. T_{reg} are a unique subpopulation of helper T-cells [89], which develops mostly in the thymus, but can also be generated in the periphery, upon TCR stimulation of naïve T cells in the presence of transforming growth factor β (TGFβ) [90]. These cells are mostly characterized by the expression of the surface molecules CD4 and CD25, and the transcription factor forkhead box P3 (FOXP3). T_{regs} have been proven to have an essential role in immune self-tolerance, as mice and humans lacking FOXP3 develop a very strong autoimmune-like lymphoproliferative disease [91-93].

T cell metabolism

Throughout the development stages mentioned above, T cells suffer several metabolic alterations that strongly contribute to its differentiation, function, fate and signalling regulation. Quiescent (naïve and memory) and activated (effector) T cells relay on different sources of energy [94-96]. Quiescent T cells present a catabolic metabolism, with nutrients being broken down to fuel the cell necessities. In contrast, activated T cells are characterized by an anabolic metabolism, where nutrients are used for macromolecular synthesis. The balance between catabolic and anabolic pathways determines the amount of adenosine triphosphate (ATP) produced and consumed by the cell [94].

TCR stimulation and CD28 co-stimulation are associated with the transport of the glucose transporter 1 (Glut1), which regulates glucose uptake, to the cell surface [97, 98]. Growth factor cytokines also further increase Glut1 expression [99-101]. It is clear that glucose is an essential nutrient, as complete deprivation of glucose impairs cytokine production, survival capacity and proliferation of T cells [102-104]. While naïve T cells low energy demands depend on oxidation of glucose trough oxidative phosphorylation (OXPHOS), upon activation T cells breakdown glucose through anaerobic glycolysis, which fuels the production of ATP essential to support cell proliferation (Fig.3) [94, 105]. In addition, amino acids breakdown is also required for proliferation [106]. In this context, it should be highlighted the importance of mitochondria. During activation, glutaminolysis a mitochondrial pathway where glutamine is converted into α-ketoglutarate, is

upregulated. It has been shown that repression of glutaminolysis impairs T cell cytokine production and proliferation [107]. T cells highly rely on healthy and functional mitochondria to properly respond to stimulus and provide protection against threats to the organism such as infection, tumor cells or chronic inflammation. Mitochondria also support cell proliferation and growth by providing building blocks for macromolecule synthesis [108]. In addition to these alterations, during activation there is a significant increase of mitochondrial mass in T cells, indicating increased biogenesis, which is indispensable for T cells to escape from quiescence [109, 110].

The pool of memory T cells established after clearance of effector T cells suffers a conversion back to mitochondrial OXPHOS (Fig.3) [94, 111, 112]. These memory T cells have now an increase in OXPHOS capacity, having high spare respiratory capacity (SRC), meaning that they are able to produce extra energy when under stress. This allows them to proliferate more quickly, maintain higher ATP levels and produce more cytokines in a situation of re-exposure to known antigens [113].

In the case of exhaustion, the T cell metabolism is characterized by a decrease in glycolysis and glucose uptake, mitochondrial dysregulation which is accompanied by enlargement and depolarization of the mitochondria and increased reactive oxygen species (ROS) production [114]. The repression of peroxisome proliferator-activated receptor-gamma coactivator (PGC1)-α expression, a master regulator of mitochondrial biogenesis, by PD-1 highly contributes to these alterations [114, 115].

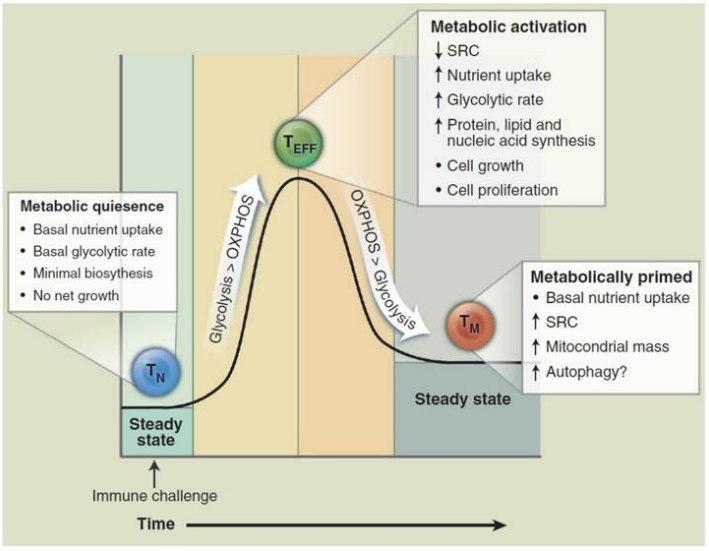


Figure 3: Metabolic alterations throughout T cell differentiation. Naïve T cells (T_N) are in a quiescence state, characterized by basal energetic needs. After activation T cells, now effector cells, (T_{EFF}) switch from oxidative phosphorylation (OXPHOS) to manly glycolysis, which fuels the increased demand for ATP production to support cell growth and proliferation. The pool of memory T cells (T_M) established after clearance of effector T cells, suffers a conversion back to mitochondrial OXPHOS. This subset has now a higher mitochondrial mass and spare respiratory capacity (SRC), meaning that they are able to produce extra energy when under stress. In addition, induction of autophagy has been shown to contribute to sustained T cell viability after activation [116, 117], raising the hypothesis that autophagy contributes for the differentiation of memory T cells. Image from *Pearce et al* [118].

Immunosenescence

Aging-induced T cell alterations

Aging is an unavoidable biologic process, characterized mainly by the progressive loss of physical fitness, resulting in increased vulnerability to environmental challenges [119]. Aging surges as a consequence of several different factors, such as the accumulation of genetic damage throughout life, epigenetic alterations, impaired protein proteostasis, cellular senesce and metabolic and mitochondrial dysfunction [120-125]. The complex dynamics between all contributing factors and how they interact within and between tissues and organs is a barrier to identify truly age-related changes. To address this, recent studies have focused on performing whole-organism analysis of ageing [126-128].

Aging of the immune system or immunosenescence decreases the ability of the organism to provide an effective response to pathogens, contributing to an increased mortality rate [129, 130]. T cells are known to suffer major age-dependent alterations, that gradually compromise their functions, which very possibly contributes to the increased susceptibility to developing autoinflammatory and autoimmune diseases, as well as to insufficient responses to vaccination, later in life [131]. One of the most significant consequences of aging is the reduction of naïve T cells and increase of frequency of effector memory T cells, which are often dysregulated [132-134]. Aged-

associated T cells directly contribute to the exacerbated inflammation and promote senesce of close and distant cells by producing cytokines such as IFNγ and TNF [57]. There is loss of surveillance function by T cells, leading to an accumulation of damaged cells [135]. The increased cytotoxicity of senescent CD8 and CD4 T cells during ageing can directly damage cells in the tissues [136, 137]. In addition, T cells can also contribute to age-associated diseases by inducing alterations in the gut homeostasis (Fig.4) [138, 139]. Age-associated T cell subsets have been shown to possess metabolic alterations, such as loss of sirtuin (SIRT) 1, which is a protein deacetylase that regulates the activation of several transcription factors, which in turn regulate metabolism [140]. Loss of SIRT1 has been associated with diminished mitochondrial capacity and increased cellular senescence [141]. It has also been reported that in aged cells, naïve CD8 T cells presented increased mitochondrial mass, but a loss of mitochondrial respiratory capacity, which was thought to be driven by reduced expression of electron transport chain (ECT) associated genes [142]. In this context, senescent mouse stem cells supplementation with a coenzyme for redox reaction, nicotinamide adenine dinucleotide (NAD^+), contributed to mitochondrial recover and cellular function improvement through a SIRT-1 dependent mechanism [141].

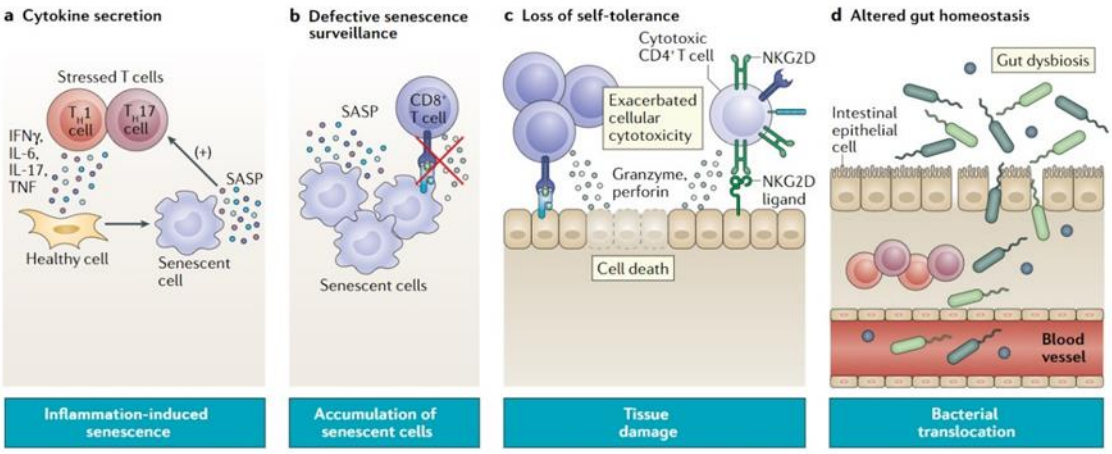


Figure 4: T cell contribution to inflammaging at the molecular level. Aged-associated T cells have increased production of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF), exacerbating inflammation and promoting senesce of other cells. There is loss of surveillance function, leading to an accumulation of damaged and senescent cells. The increased cytotoxicity of senescent cells can directly damage cells or the tissues due to their loss of self-tolerance. T cells can also contribute to age-associated diseases by inducing alterations in the gut homeostasis. Image from *Carrasco et al* [143].

On a transcriptional level, several studies have tried to identify an aging transcriptional signature [134, 144-147]. They have revealed that there is an accentuated cell to cell transcriptional variability in CD4 T cells of older mice, meaning that they have more scattered transcriptional responses [144]. A large-scale single-cell RNA sequencing analysis of the CD4 T cell population in young and adult mice showed that this compartment is characterized by an increase of cytotoxic and exhausted subsets in older mice [134]. All together, these recent reports indicate that ageing both in humans and mice is characterized by a differentiation of T cells into an activated and cytotoxic state, which is a key contributor to the increased inflammation observed during ageing, which is referred as inflammageing. In this setting, it is important to understand if IL-10 is able to activate T cells and induce an inflammatory signature in a similar way as the ageing process. The described cell alterations developed during ageing are assumed to be linked to the thymus involution, cellular senescence processes and lifelong antigen encounters [130]. For the purpose of this thesis, I will only focus on the process of T cell senescence.

Characteristics of senescent T cells

In older humans, memory T cells were found to transition into senescent or exhausted cells as they lose the expression of co-stimulatory molecules such as CD28 and CD27 [143]. Functionally, senescent T cells secrete more pro- and anti-inflammatory cytokines, while exhausted T cells supposedly lose their ability to produce effector cytokines [143]. However, it should be mentioned that a recent report showed that a subset of exhausted-like CD8 T cells accumulated in tissues, in both older humans and mice, and exacerbated the inflammation [147].

Senescent T cells are characterized by increased expression of markers such as TIM-3, CD57, CD45RA, killer cell lectin-like receptor subfamily G member (KLRG-1) [148-150] and p16, p21 and p53, which are cell cycle regulatory proteins [151, 152]. Senescent T cells are also very commonly identified through their senescence-associated secretory phenotype (SASP), as they produce several proinflammatory cytokines, such as IL-8, IL-6, IFNγ and TNF-α [131, 152-154]. This secretion is kept due to the presence of DNA segments with chromatin alterations reinforcing senescence – DNA-SCARS [155]. Senescent T cells present features commonly associated with ageing such as DNA damage markers, which includes phosphorylation of histone H2AX (γH2AX) and high activity of senescence-associated β-galactosidase (SA-β-gal) [132]. Elderly individuals present an increased frequency of senescent T cells [131, 132], phenotype that might be

induced by T_{regs} as a mechanism of suppression of naïve and effector T cells [151, 156]. Senescent T cells are thought to be highly involved in age-related diseases, as it was even shown that T cells derived from old mice present an immunosenescence phenotype [157]. Cardiovascular and metabolic disorders, which are very common in aged individuals, have been associated with overall increased T cell senescence and with T cell alterations in the adipose tissue [158-162]. T cells with a $CD4^{+}CD44^{hi}CD62L^{lo}PD\text{-}1^{+}CD153^{+}$ phenotype, which are described as senescent T cells, were shown to accumulate in visceral adipose tissue in obese mice and during aging, where they contribute to chronic inflammation in the tissue [163-165]. The adipose tissue, as the largest energy storage organ, plays a fundamental role in the metabolism homeostasis of other organs and tissues through several pathways [166]. Proinflammatory cytokines originated from the adipose tissue contribute very strongly to systemic inflammation, impacting the rest of the organism [126, 167]. All of this supports the idea that senescent T cells strongly contribute to inflammation during ageing and its consequent negative impact in tissues and organs. Therefore, T cell-based therapies are emerging as possible approaches to delay the development of age-associated disorders [143].

Objectives

Given the important role of IL-10 in inflammatory responses and its potential for inflammatory diseases treatments, it is critical to fully understand the impact of IL-10 at the cell and organism level. Unveiling IL-10 mechanisms of action can help to better understand the recently described non-classical roles of this cytokine, including by our group. Ultimately it will also open new perspectives for IL-10 based treatments. In this context, the specific aims of my thesis were to:

1. Fully characterize the T cell alterations in response to *in vivo* exposure to IL-10.
2. Understand the impact of IL-10-reprogrammed T cells in the organism.
3. Understand the mechanisms underlying the IL-10-mediated T cell reprogramming.

Methods

Ethics Statement

All animal experiments were performed in strict accordance with recommendations of the European Union Directive 2010/63/EU and previously approved by Portuguese National Authority for Animal Health-*Direção Geral de Alimentação e Veterinária* (DGAV). The Guide for the Care and Use of Laboratory Animals, principle of the Three R's, to replace, reduce, and refine animal use for scientific purposes, as well as FELASA recommendations were used. Mice were euthanized by CO_2 inhalation with effort to minimize suffering. I obtained the FELASA B certification for laboratory animal work.

Mice

Wild type C57BL/6j, pMT10 mice, pMT-10 crossed with IFN-γ deficient mice (pMT-10.IFN-$\gamma^{-/-}$), pMT-10 crossed with IL-10Rα deficient mice (pMT-10. IL-10R$\alpha^{-/-}$) and pMT-10 crossed with CD3 deficient mice (pMT-10.CD3$^{-/-}$) were bred either at i3S (Portugal) or the Pasteur Institute (France) under specific pathogen-free conditions. All animals were maintained with food and water ad libitum, on a 12:12 light cycle at 45-65% humidity and environment temperature maintained between 20ºC and 24ºC.

IL-10 induction

IL-10 over-expression is induced in pMT-10 mice by feeding the mice with drinking water composed by 2% sucrose (PanReac AppliChem, Germany) and 50 mM of Zn sulphate heptahydrate (Sigma-Aldrich, MO, USA) [40]. Control C57BL/6j fed with the Zn solution do not upregulate IL-10 [40].

Bone Marrow Transplantation Assays

Chimeric mice were generated by transferring sorted bone marrow LSK cells from pMT-10 or pMT-10. IL-10R$\alpha^{-/-}$ mice into sub lethally irradiated (400 rad) pMT-10.CD3$^{-/-}$ recipient mice. Transplantation was performed by intravenous administration of 2000-5000 cells in 0.2 mL of phosphate-buffered saline solution (PBS) (GIBCO). After 6 weeks, 0.1 mL of blood were collected from each reconstituted mouse to assess the efficacy of reconstitution.

Preparation of cell suspensions

Femurs, tibias, and spleens were collected to obtain cell suspensions. BM cells were extracted according to an already published protocol [168]. Spleens were mechanically disaggregated. Briefly, cell suspensions were recovered into PBS supplemented with 2% FBS. Cell suspensions were incubated with ammonium-chloride-potassium (ACK) lysing

buffer for 5 minutes to lyse red blood cells. Cells were then filtered with 40 or 70 µm cell strainers.

Flow Cytometry

To assess efficacy of reconstitution in chimeric mice, blood samples were stained with CD3 BUV397 (1:100), CD19 APC (1:200) and either IL-10Rα PE (1:100) or its respective isotype control (all from Biolegend). After a 30 minutes incubation at 4ºC with the antibodies, cells were resuspended in 0.5 mL of lysis buffer diluted 1:10 in H_2O. Samples were washed twice with PBS supplemented with 2% fetal bovine serum (FBS) and then acquired on LSRFortessa (BD Biosciences).

For T cell surface markers analysis, cells were incubated with FcBlock (1:200) for 10 minutes at 4ºC, they were then stained with viability dye, Zombie Aqua (1:500), for 20 minutes at RT, followed by a 20 minutes incubation at 4ºC with CD3 PerCP-Cy5.5 (1:100), TCRβ APC-Cy7 (1:200), CD4 BV785 (1:200), CD62L PE (1:200), PD-1 BV421 (1:200), CD44 PE-Cy5 (1:2000), CD8 PE-Cy7 (1:300) (all from Biolegend).

For yH2AX marker analysis, cells were incubated with CD3 PerCP-Cy5.5 (1:100), TCRβ APC-Cy7 (1:200), CD4 BV785 (1:200), and CD8 PE-Cy7 (1:300) (all from Biolegend) for 20 minutes at 4ºC. They were then incubated with fixation buffer (eBioscience™) for 10 minutes at RT, followed by a 15 minutes incubation with 1X Permeabilizing/Wash buffer (eBioscience™) and a 60 minutes incubation with yH2AX APC (1:100) (Biolegend) diluted in 1X Permeabilizing/Wash buffer.

For mitochondrial dyes staining, cells were incubated with 30 minutes at 37ºC with MitoTracker™ Green FM (50 nM) and MitoTracker™ Red CMXRos (50 nM) (all from Invitrogen™) in RPMI-1640 medium (GIBCO), followed by a 20 minutes incubation at 4ºC with CD4 PerCP (1:100) and TCRβ PE-Cy7 (1:200) (all from Biolegend).

Stained cells were analyzed by multiparametric flow cytometry. Samples were acquired on LSR Fortessa (BD Biosciences). Data were analyzed on FlowJo software.

Cell Sorting

BM cell suspensions were incubated for 20 minutes at 4ºC with biotinylated antibodies (CD3, CD4, CD8, CD11b, CD11c, Gr1, CD19, B220, NK1.1, TER-119, TCRβ, TCRγδ) (Biolegend), followed by a 15 minutes incubation at 4ºC with anti-biotin microbeads (Miltenyi Biotec). Enrichment of target population was then performed with magnetic separation (MACS LS columns, Miltenyi Biotec), through positive selection. After enrichment cells were stained with Sca-1 BV510 (1:400), cKIT APC-Cy7 (1:200), CD3

PE (1:500) and streptavidin BV785 (1:200) (all from Biolegend). Cells were resuspended in PBS supplemented with 2% FBS and propidium iodide (1:4000) (Invitrogen™). Samples were purified on FACSAria III (BD Biosciences).

X-ray micro-computed tomography (MicroCT)

X-ray micro-computed tomography (Bruker SkyScan1276) was performed by the Bioimaging unit at i3S to obtain 3D images of the animals' internal microstructure. All images were analyzed on the NReconstruction and CT-Analyser software to quantify total body fat.

Histology

Gonadal white adipose tissue was collected from mice. For histopathology, the harvested tissues were fixed in 10% formalin for 48 hours at room temperature and were then embedded in paraffin and cut into 3 µm sections. Sections were then deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E), according to standard techniques. Images were acquired on an optical microscope using a DP 25 Camera and Software Cell B (Olympus, NY, USA) at 10x magnification. Adipocyte area was quantified using the plug-in "Adiposoft" in the ImageJ/Fiji software. From each animal between 518 to 891 adipocytes were measured from 2-4 different images of the same section.

Senescence-associated β-galactosidase (SA-β-gal)

β-Galactosidase staining was performed with the Senescence β-Galactosidase Staining Kit (Cell Signaling) following the manufacturer's instructions. Briefly, adipose tissue was transferred into 24-well plates and coated with 0.5 mL of β-Galactosidase staining solution with a pH of 5.9 to 6.1. The plate was incubated at 37ºC in a dry incubator (no CO_2) for 2-3 hours.

Statistical analysis

Statistical significance was determined using Student t test, one-way analysis of variance (ANOVA) or two-way ANOVA. These tests were performed with Prism Software (GraphPad). Statistical significance is represented as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Results

In vivo IL-10 exposure reprograms CD4 and CD8 T cells towards an activated aged-like state

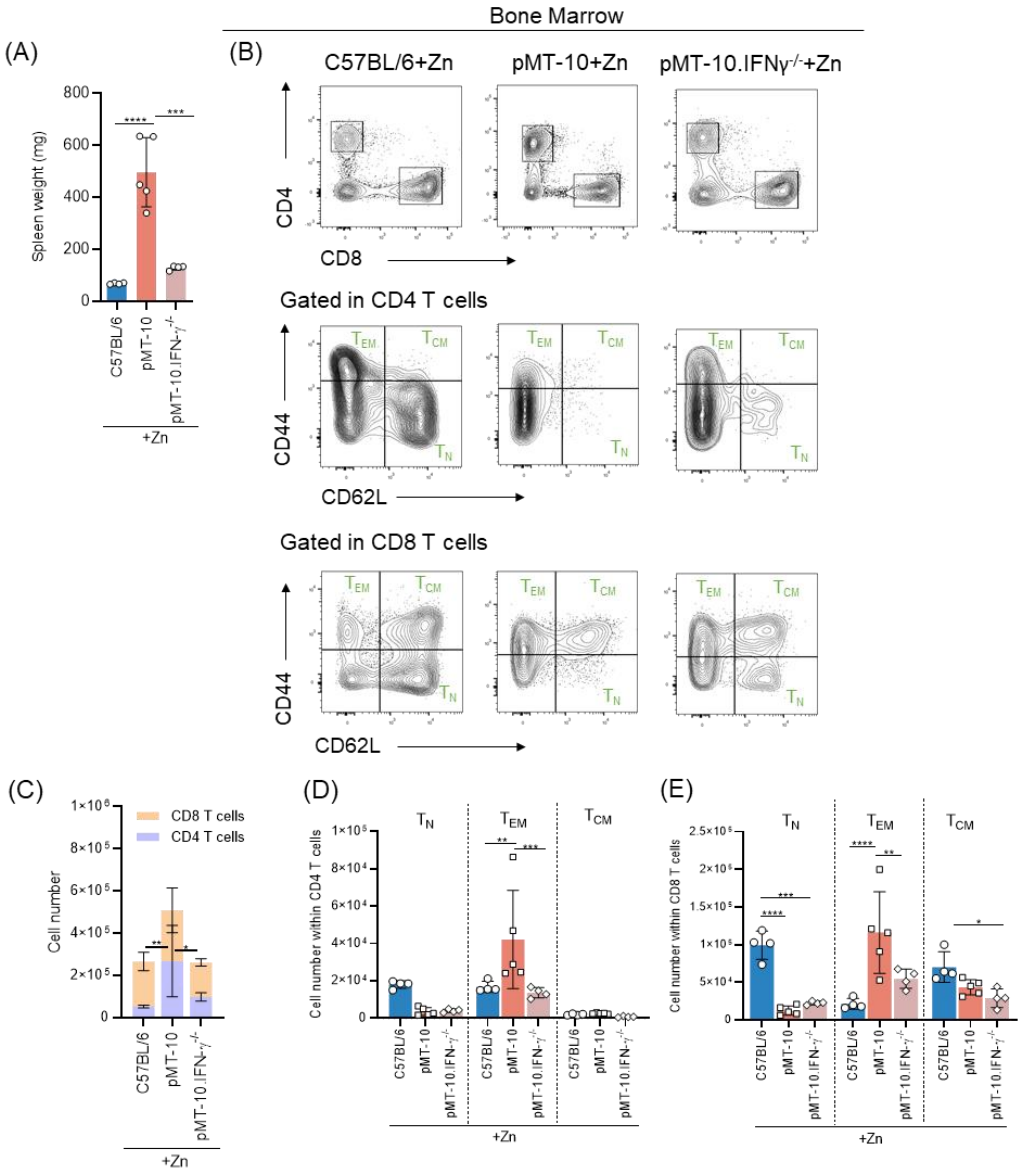
In our group previous report [41], targeted RNA sequencing (RNA-seq) in sorted CD4 and CD8 BM T cells showed that IL-10 led to a transcriptional reprogramming, with 568 and 202 genes differentially expressed in CD4 and CD8 T cells, respectively. Control mice, C57BL/6 fed with zinc and non-induced pMT-10 did not present this transcriptional reprogramming. A more detailed analysis showed an upregulation of senescence and exhaustion-associated genes in both CD4 and CD8 T cells isolated from the BM of pMT-10 induced to overexpress IL-10 for 30 days versus control mice (C57BL/6 mice fez with Zn) and non-induced pMT-10 mice. In CD4 T cells we observed an increased expression of *Cdkn2a (p16)*, *Cdkn1a (p21), Gadd45 (growth arrest and DNA damage inducible alpha)*, which are genes associated with senescence and DNA damage. In CD8 T cells there was a greater expression of genes associated with exhaustion such as *Pdcd1*, which encodes PD-1, *Ctla-4* and *Lag-3 [169, 170].* In addition, in both populations there was an increase of SASP-associated genes, such as *C-C Motif Chemokine Ligand (Ccl) 5*, *Ccl3* and *Ccl4 [154]*. Further re-analysis of our previous RNA-sequencing data (data not shown) revealed that the transcriptome signature of CD4 T cells from mice exposed to IL-10 follow the same pattern of cytotoxic activation as CD4 from aged mice described by Elyahu et al [134]. After finding these transcriptional alterations, we questioned if T cells could be activated by IL-10 in a similar way as it occurs during ageing.

To address this question, we started by investigating the phenotype of BM and spleen CD4 and CD8 T cells during IL-10 induction. For this, we induced IL-10 over-expression in pMT-10 mice for 30 days, through administration of Zn in the drinking water. To understand which alterations are dependent of IFN-γ, we also analysed pMT-10 mice deficient for IFNγ (pMT-10.IFNγ$^{-/-}$) fed with Zn. As controls, we used C57BL/6 mice fed with Zn.

Given that induction of IL-10 leads to splenomegaly due to an expansion of the splenic myeloid compartment [41], we started by weighting the mice spleens after the 30 days of IL-10 induction. As expected, a pronounced increase in spleen weight was observed in pMT-10 mice fed with Zn. This alteration was not present in C57BL/6 nor in pMT-10.IFNγ$^{-/-}$ mice fed with Zn (Fig.5A). In the BM, we found alterations to the CD4/CD8 T cells ratio in pMT-10 mice overexpressing IL-10, which displayed an increase in the number of CD4 T cells as compared to control C57BL/6 mice (Fig.5B,C). Furthermore, in pMT-10 mice, IL-10 led to a reduction of the T cell naïve compartment (T_N, CD44$^-$ CD62L$^+$), which was accompanied by an expansion of the T cell effector memory population (T_{EM}, CD44$^+$CD62L$^-$) both in CD4 (Fig.5B,D) and CD8 (Fig.5B,E) T cells. The

central memory compartment (T_{CM}, $CD44^{+}CD62L^{+}$) did not suffer significant alterations (Fig.5B,D,E). In the absence of IFNγ, IL-10 induction did not result in alterations to the ratio of CD4/CD8 T cells (Fig.5C). However, In the CD4 T cell compartment pMT-10.IFNγ$^{-/-}$ mice also presented a decrease of naïve cells but no alterations in the effector compartment (Fig,5B,D). Within the CD8 T cell compartment, pMT-10.IFNγ$^{-/-}$ mice showed similar alterations to IL-10 induced pMT-10 (Fig.5B,E).

The spleen T cell pool revealed no difference in the ratio CD4/CD8 in pMT-10 mice exposed to IL-10 (Fig.5F,G). Within the CD4 T cell population there was a significant increase of naïve T cells (Fig.5F,H). However, there were no significant alterations in the effector compartment (Fig.5F,H). In the CD8 T cell compartment these alterations were not statistically significant (Fig.5F,I). When compared to pMT-10 mice, pMT-10.IFNγ$^{-/-}$ mice behaved similarly (Fig.5F-I).



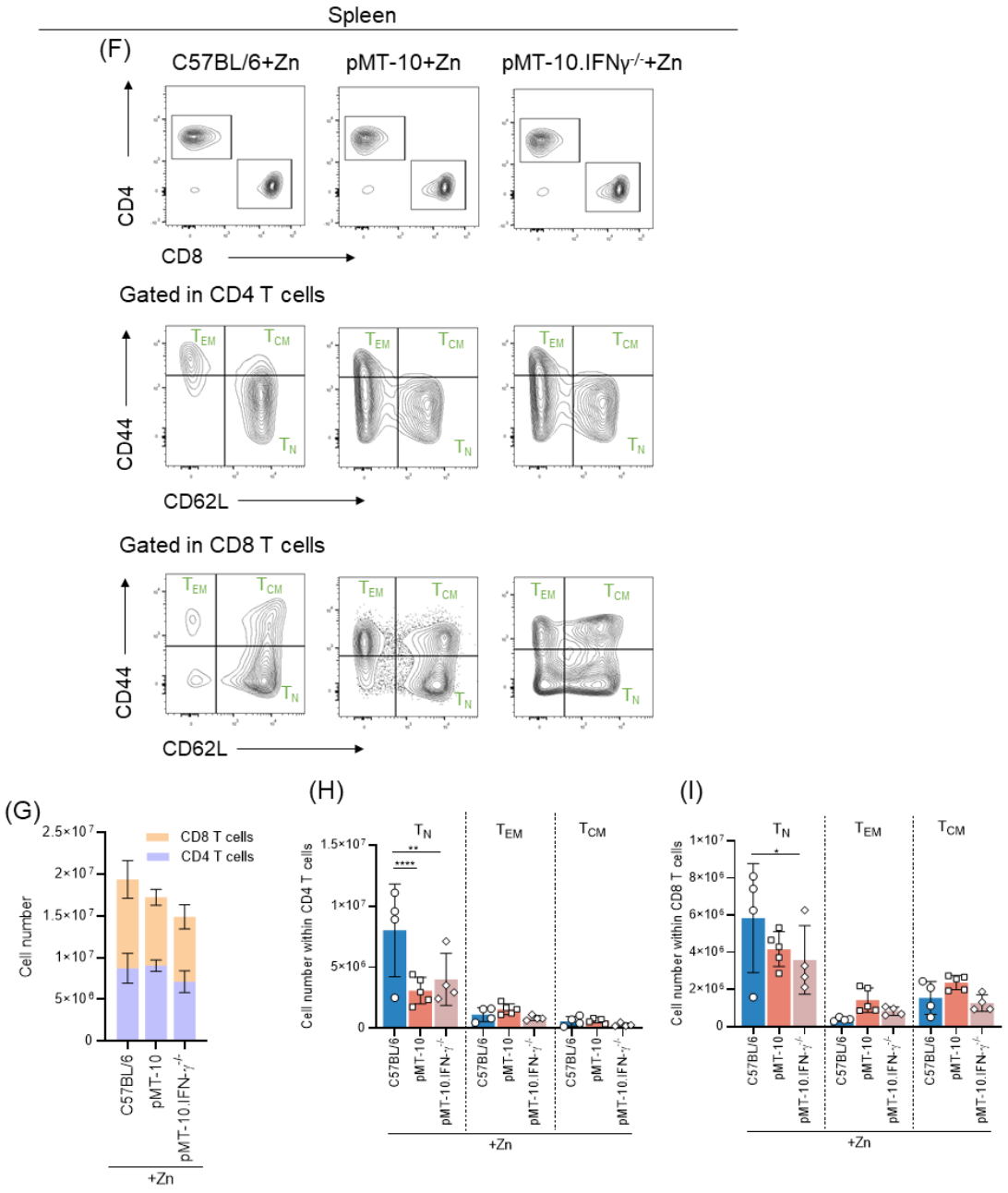


Figure 5: IL-10 induces alterations in bone marrow and spleen CD4 and CD8 T cell populations. Mice of the indicated genotype were fed with zinc for 30 days, (A) total spleen weight. Cell suspensions of bone marrow and spleen were analyzed by flow cytometry. (B,F) Representative plots. (C,G) Number of CD4 and CD8 T cells and number of cells in each compartment - naïve (T_N), effector memory (T_{EM}) and central memory (T_{CM}) - within (D,H) CD4 and (E,I) CD8 T cells in the (B-E) bone marrow and (F-I) spleen. Each bar represents the mean ± SD and each dot represents one animal, in one of two independent experiments. Statistical differences were calculated by one-way ANOVA (A) or two-way-ANOVA (C-E,G-I). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

We then questioned if PD1 and CD38 expression would be altered in T cells upon IL-10 induction, as these markers have been previously linked to T cell senescence and exhaustion [171, 172], as well as being detected as differentially expressed upon IL-10 exposure in our RNA-Seq analysis of CD4 and CD8 T cells [41]. As compared to controls, we found an accentuated increased expression of PD-1 and CD38 in pMT-10 mice over-

expressing IL-10 in both BM (Fig.6A,B) and spleen (Fig.6C,D) CD4 and CD8 T cells, indicating high levels of cell activation. Interestingly, although less accentuated, BM and spleen T cells from pMT-10.IFNγ$^{-/-}$ also presented an increased expression of these markers (Fig.6A-D).

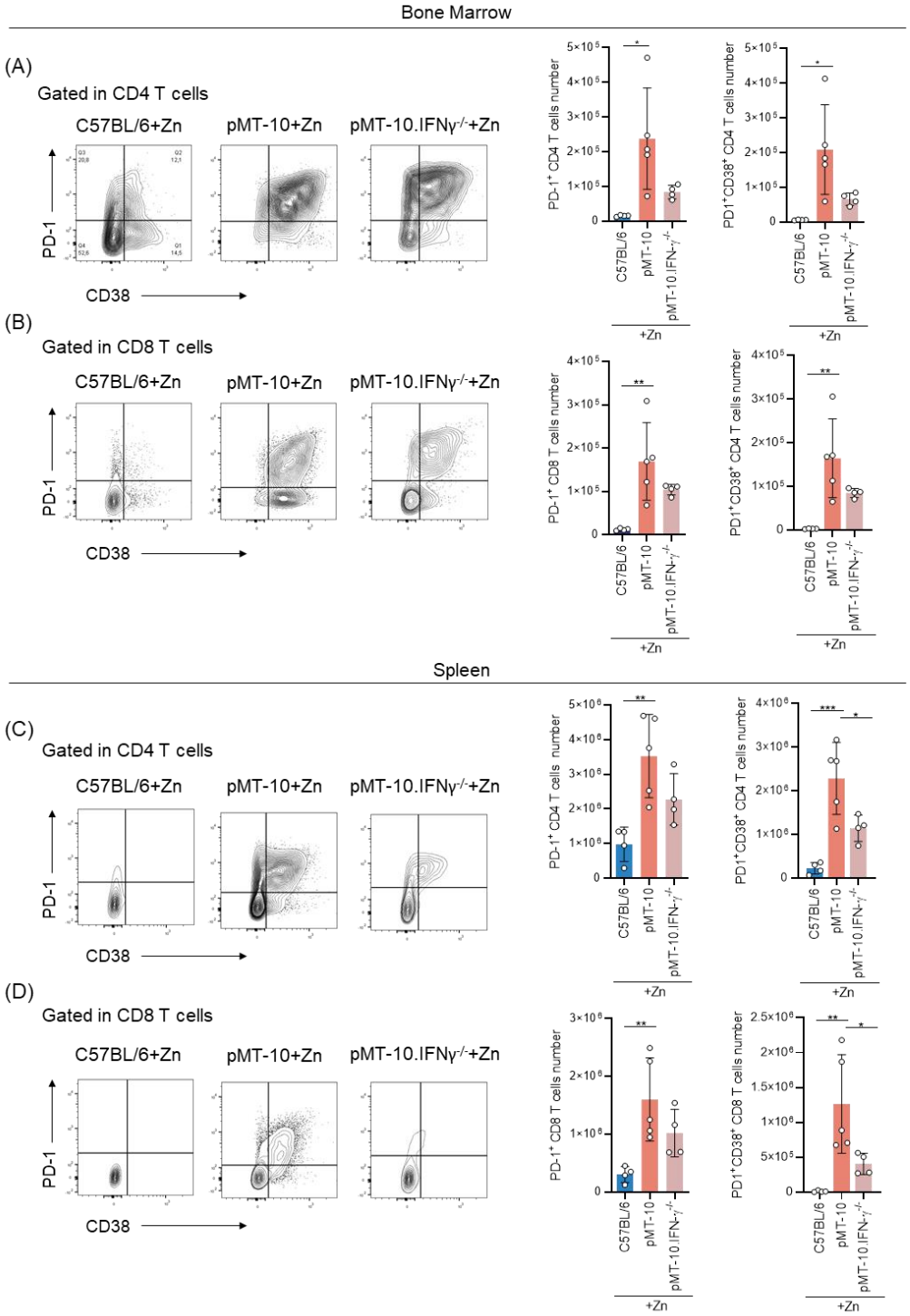


Figure 6: IL-10 increases the expression of CD38 and PD1 surface markers in CD4 and CD8 T cells. Mice of the indicated genotype were fed with zinc for 30 days, and cell suspensions of bone marrow and spleen were analyzed by flow cytometry. Representative plots and number of PD1$^+$ and PD1$^+$CD38$^+$ cells within (A,C) CD4 and (B,D) CD8 T cells in the (A-B) bone marrow and (C-D) spleen. Each bar represents the mean ± SD. and each dot represents one animal, in one of two independent experiments. Statistical differences were calculated by one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.

In vivo exposure to IL-10 increases the frequency of dysfunctional T cells

The marked reduction of the naïve T cell compartment, increased number of effector T cells and, particularly, the accumulation of a $PD1^{+}$ population, together with the transcriptional signature compatible with exhausted and senescent T cells, suggest that *in vivo* IL-10 exposure may drive the differentiation of aged T cells. With this hypothesis in mind, we decided to assess T cell functionality through different parameters. As mitochondrial dysfunction has been associated with impaired T cell functionality [173], we started by verifying if the mitochondrion of these CD4 and CD8 T cells were healthy. For this, we analysed two mitochondrial dyes through flow cytometry analysis, MitoTracker green (MTG) and MitoTracker Red (MTR). MTG is a mitochondria-selective dye insensitive to membrane potential, therefore it labels total mitochondria mass, both healthy and unhealthy mitochondria [174]. MTR is dependent of mitochondrial membrane potential, being used to quantify gain or loss of mitochondrial functionality [114]. The population $MTG^{high}MTR^{high}$ represents functional mitochondria and the population $MTG^{high}MTR^{low}$ indicates depolarized mitochondria, which are considered dysfunctional [175]. To evaluate the ratio of functional and dysfunctional mitochondrion in the BM and spleen, we induced pMT-10 mice with Zn for 30 days. As controls we used C57BL/6 fed with Zn. We observed a decrease of functional mitochondrion ($MTG^{high}MTR^{high}$) in CD4 T cells, both in BM (Fig.7A) and spleen (Fig.7B), indicating that IL-10 induced mitochondrial dysfunction in CD4 T cells. We are currently planning to repeat this experiment to also include CD8 T cell analysis.

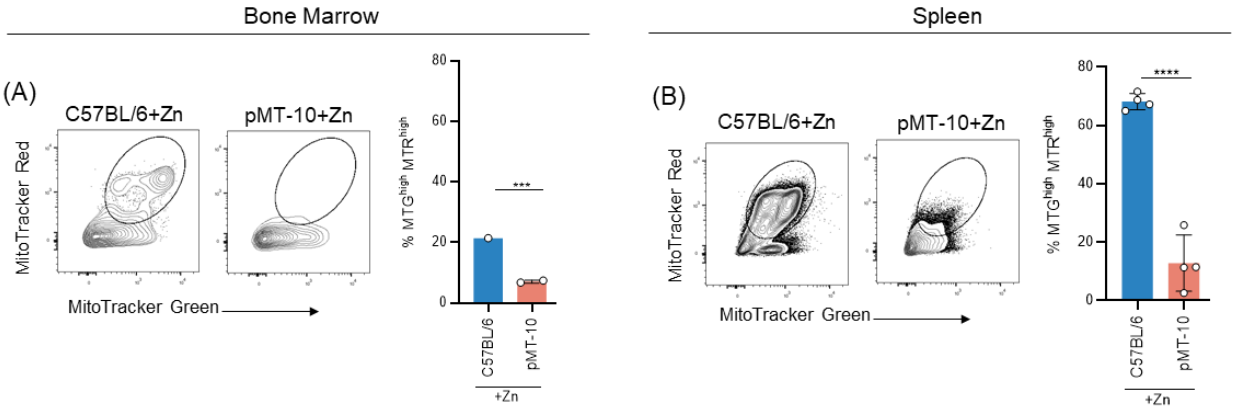


Figure 7: IL-10 induces mitochondrial dysfunction in CD4 T cells. Mice of the indicated genotype were fed with zinc for 30 days, and cell suspensions of boné marrow and spleen were analyzed by flow cytometry. Representative plots and frequencies of functional mitochondrion ($MTG^{high}MTR^{high}$) in CD4 T cells from the (A) bone marrow and (B) spleen. Each bar represents the mean ± SD and each dot represents one animal, in one experiment. Statistical differences were determined by Student´s t test. **p<0.001, ****p<0.0001.

Given the fact that DNA damage, which accumulates with age, is a mediator of cellular senescence, we questioned if there would be an accumulation of DNA damage in T cells from mice over-expressing IL-10. For this, we analysed the expression of γH2AX, a common DNA damage marker [132]. Indeed, *in vivo* IL-10 exposure led to an increase of γH2AX positive CD4 and CD8 T cells isolated from the BM (Fig.8). These results are in agreement with the alterations previously described, reinforcing the negative impact of IL-10 in T cells as it induces age-like features.

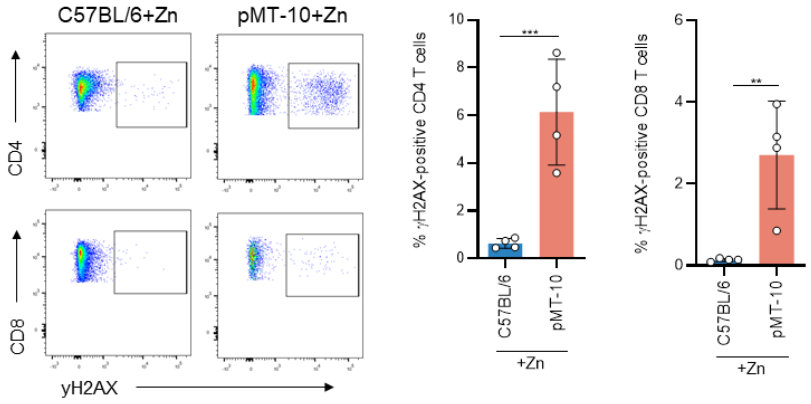


Figure 8: IL-10 induces DNA damage in bone marrow CD4 and CD8 T cells. Mice of the indicated genotype were fed with zinc for 30 days, and cell suspensions of bone marrow were analyzed by flow cytometry. Representative plots and percentages of γH2AX-positive CD4 or CD8 cells in the bone marrow. Each bar represents the mean ± SD and each dot represents one animal, in one experiment. Statistical differences were calculated by Student´s t test. **p<0.01, ***p<0.001.

IL-10 induces senescence in the adipose tissue

T cells with dysfunctional mitochondria have been shown to initiate a strong inflammatory program that triggers the development of various characteristics associated with ageing, at the organism level [176]. Considering that the adipose tissue is one of the most studied tissues in the context of aging, we next investigated the impact of IL-10 induction to this tissue. For that, we harvested gonadal white adipose tissue from pMT-10 or control mice fed with Zn. We started by performing a histological analysis by H&E staining of the adipose tissue of pMT-10 and C57BL/6 mice fed with Zn for 30 days. We found that mice induced with IL-10 displayed a decrease in the size of their adipocytes (Fig.9A). It was also noticeable that the adipose tissue from pMT-10 mice presented inflammatory infiltrations, which are known as crown-like structures (Fig.9A). These structures are histological hallmarks of proinflammatory processes in adipose tissue seen in aged tissue [177]. To further address this alteration, we analysed different immune cell populations in the adipose tissue of pMT-10 mice over-expressing IL-10 and C57BL/6 as control mice. After being exposed to high levels of IL-10, mice showed an accumulation of T cells ($CD3^+$), both CD4 and CD8 T cells, and an accentuated decrease

in myeloid (CD11b$^+$) and B cells (CD19$^+$) (Fig.9B). This suggests that circulating T cells may be accumulating in the adipose tissue, after *in vivo* IL-10 exposure. As we showed that IL-10 reprogrammed T cells present features compatible with aged T cells and because aged T cells may cause tissue senescence [163].we next assessed cellular senescence in the adipose tissue. For that, we did a staining for SA-β-gal activity, a widely used assay to detect tissue senescence [178]. We found SA-β-gal activity in the adipose tissue of pMT-10 mice fed with Zn, whereas no staining was observed in the adipose tissue of C57BL/6 (Fig. 9C). This result suggests that IL-10 induction leads to increased senescence in the adipose tissue.

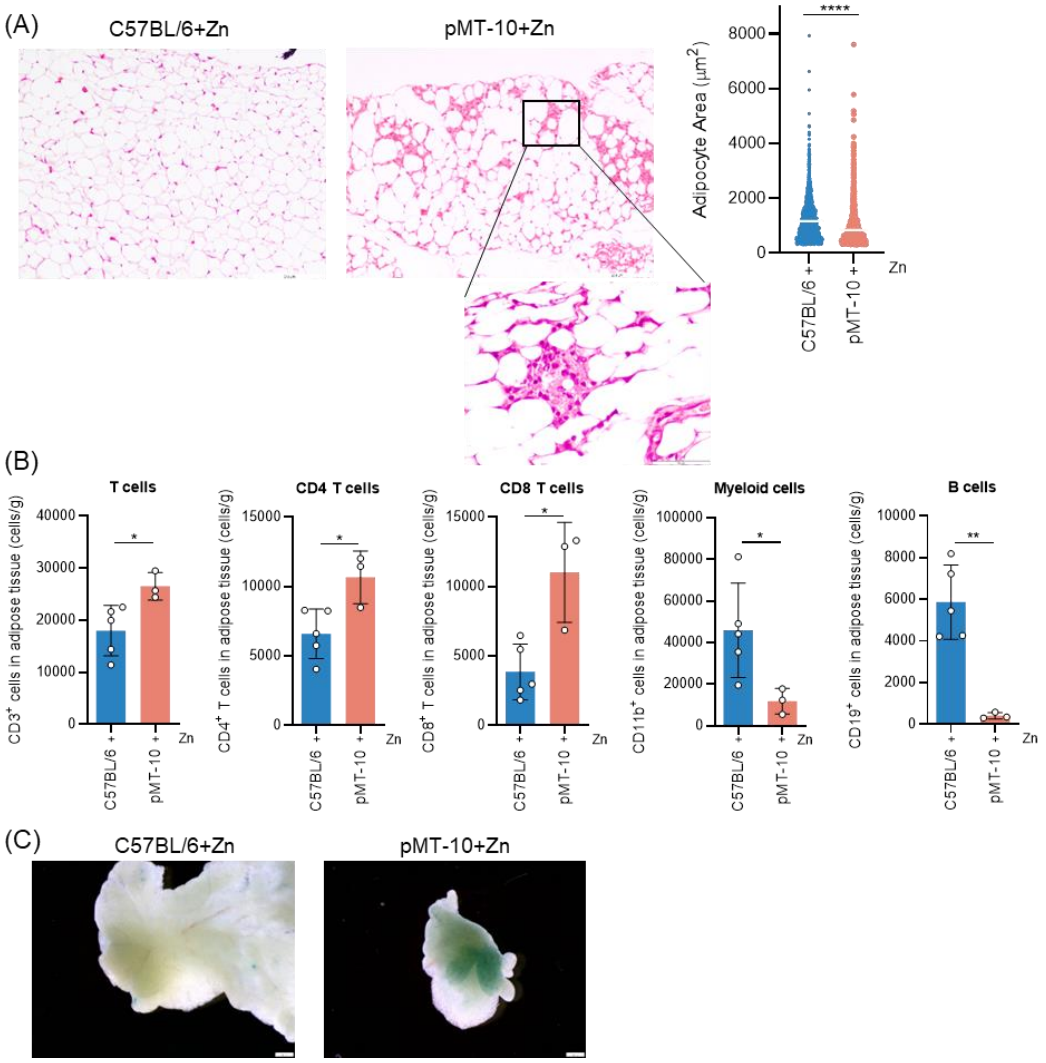


Figure 9: IL-10 induces structural and immune cell alterations in the adipose tissue. Mice of the indicated genotype were fed with zinc for 30 days, and their gonadal white adipose tissue was analyzed. (A) Representative H&E-stained sections of adipose tissue at 10x or 40x magnification (Scale bar, 200 µm or 50 µm) and adipocyte area quantification. The bar represents the mean and each dot represents one adipocyte. From each animal adipocytes were measured from 2-4 different images of the same section and 3-4 animals per group were used, in one experiment. Cell suspensions were prepared, stained and analyzed by flow cytometry. (B) Number of CD11b$^+$, CD19$^+$ and CD4 and CD8 T cells in the adipose tissue. Each bar represents the mean ± SD and each dot represents one animal, in one experiment. (C) Representative pictures of gonadal white adipose tissue were used for senescence-associated β-galactosidase (SA-β-gal) activity assessment (Scale, 1mm), 2-4 animals from each group were used in two independent experiments. Statistical differences were calculated by Student´s t test. *p<0.05, **p<0.01, ****p<0.0001.

Considering all the alterations described after 30 days of IL-10 induction, we decided to investigate the impact of this cytokine in the organism at a later time point. For this, we induced IL-10 over-expression for 75 days in pMT-10 mice. Mice were weighed every 7 days until day 70 post Zn-administration. When compared to control C57BL/6 mice, mice over-expressing IL-10 did not gained weight throughout time as it would be expected from healthy animals (Fig.10A). At day 70 a significant difference was observed in the percentage of initial weight between the two groups (Fig.10B). Considering the previously alterations found in the adipose tissue (Fig.9), we questioned if the difference in weight variation between control and IL-10-induced animals could be related to alterations in the quantity of the adipose tissue. Through microtomography (Fig.10C), we quantified the whole-body percentage of adipose tissue in induced pMT-10 and C57BL/6 mice after 75 days of Zn administration (Fig.10D). Although no significant differences between pMT-10 over-expressing IL-10 and C57BL/6 mice fed with Zn were identified, high variability was observed in control animals, suggesting the need to increase the experimental groups to accurately determine possible differences.

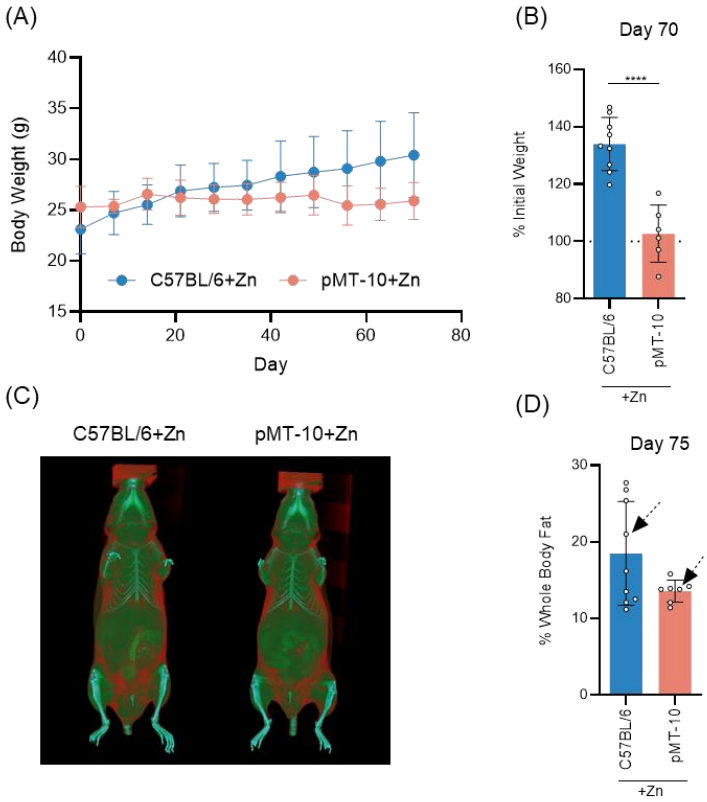


Figure 10: Mice over-expressing IL-10 do not gain weight throughout time. Mice of the indicated genotype were fed with zinc for 75 days. (A) Weight curve and (B) percentage of initial weight at day 70 of diet with zinc-enriched water. Mice were weighted at the indicated time-points. (C) Representative images of microtomography and (D) percentage of whole-body adipose tissue at day 75 post-Zn administration. Different areas are distinguished through a density gradient with red areas representing adipose tissue. Represented animals in (C) are signaled with an arrow in (D). (A,B,D) Each bar represents the mean ± SD and each dot represents one animal, in two independent experiments. Statistical differences were calculated by Student´s t test. ****p<0.0001.

Deciphering the IL-10 mechanism of action

Our results strongly suggest that therapeutical doses of IL-10 reprogram T cells towards an aged-like phenotype, with consequences at the organism level. Deciphering the pathways trough which IL-10 reprograms T cells is important to improve IL-10-mediated therapies. The first step to understand the IL-10 mechanism of action in this context is to address whether IL-10 exerts direct or indirect effects on T cells. To address this question, we performed adoptive transfer experiments. BM lineage^{-} Sca-1^{+} c-Kit^{+} (LSK) cells from pMT-10 or IL-10Rα-deficient pMT-10 mice (pMT-10.IL10Rα$^{-/-}$) were transferred into sub lethally irradiated recipient pMT-10.CD3 deficient mice (pMT-10.CD3$^{-/-}$), generating mice where all T cells are responsive (in the case of pMT-10) or unresponsive (in the case of pMT-10.IL10Rα$^{-/-}$) to IL-10 (Fig.11A). In order to evaluate if the reconstitution of chimeric mice was successful, we performed a relative quantification of the IL-10Rα in B and T cells through blood analysis 6 weeks after transplantation (Fig.11A). Indeed, B and T cells from mice reconstituted with BM cells from IL10Rα$^{-/-}$ mice lacked the IL-10 receptor (Fig.11B). This part of the experiment showed that the system was working as expected.

Reconstituted and control mice, C57BL/6 and pMT-10, were then fed with Zn for 10 days (Fig.11A). At this time point, we observed an increase in the spleen weight of pMT-10 and mice reconstituted with IL-10Rα competent cells, but not in control C57BL/6 nor in mice lacking the IL-10 receptor in T cells (Fig.11C). We then searched for possible variations on the IFNγ-producing CD4 and CD8 T cell populations in the BM. As expected, no alterations on IFNγ-producing CD4 and CD8 T cell populations were seen in control C57BL/6 mice, whereas an increase on the frequency of these cells was observed for induced pMT-10 mice (Fig.11D,E). Similarly, reconstitution of CD3$^{-/-}$ mice with IL-10R competent cells led to the expected increase in the percentage of IFNγ producing CD4 and CD8 T cells (Fig.11D,E). Finally, lack of IL-10R in T cells led to a decrease of IFNγ producing CD4 and CD8 T cells when compared with pMT-10 mice, although the frequency of these cells appears to be higher in the chimeric mice than in control C57BL/6 fed with Zn (Fig.11D,E). This finding suggest that both IL-10-dependent and independent signals may operate. To further confirm or not these results we will repeat this experiment with a higher number of mice per group.

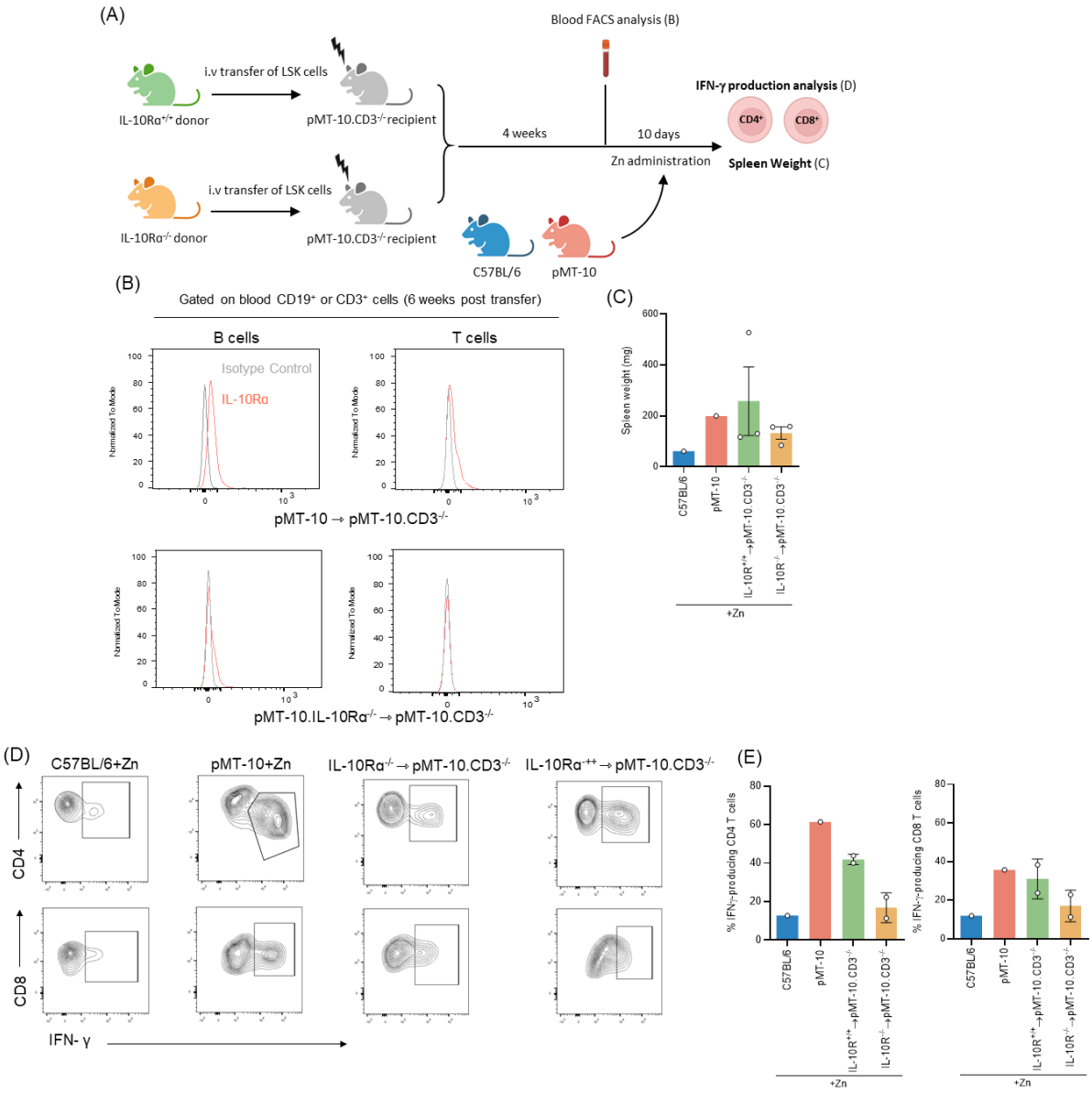


Figure 11: IL-10 may act directly and indirectly on T cells (A) Schematic figure of the experiment performed. Chimeric mice were generated by transferring sorted bone marrow lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells from pMT-10 or IL-10Rα deficient pMT-10 mice into sub lethally irradiated pMT-10.CD3 deficient recipient mice. Transplantation was performed by intravenous (i.v) administration. (B) Donor reconstitution was assessed by peripheral blood analysis 6 weeks after transplantation through flow cytometry. Mice were fed with zinc for 10 days. At this time point their spleen was weighted (C) and bone marrow analyzed by flow cytometry. (D) Representative plots and (E) frequency of interferon γ (IFNγ) producing CD4 and CD8 T cells in the bone marrow. Each bar represents the mean ± SD and each dot represents one animal, in one experiment. The limited Number of animals in each experiencial group precluded any statistical analysis

Discussion and Future Perspectives

IL-10 is a potent anti-inflammatory cytokine that ensures an appropriate immune response while preventing the appearance of immune pathology. Due to its capacity in limiting inflammatory responses, IL-10 has been tested as a treatment for inflammatory conditions, as IBD, Crohn´s disease, among others [1]. However, the administration of IL-10 was never successful in humans due to development of several side effects such as fatigue, headache, anemia, thrombocytopenia, monocytosis, neutrophilia and up-regulation of the pro-inflammatory cytokine IFNγ in the serum [31-34]. A better knowledge of IL-10 context-specific alterations and the mechanism by which it acts, may provide alternative ways to improve IL-10 clinical treatments for chronic inflammatory diseases. Even though this cytokine has been highly known for its anti-inflammatory properties, as mentioned in the introductory section, several studies have found different non-classical roles of IL-10, which are not yet fully understood. Our laboratory aims at better understanding how IL-10 can impact the organism beyond its classical anti-inflammatory capacity, and which pathways mediate the alterations imposed by this cytokine.

Our previous study showed that IL-10 exposure *in vivo* is a trigger of EM and leads to splenomegaly and anemia in mice [41]. Mice with induced expression of IL-10 display an expansion of LSKs and GMPs in both the spleen and BM. The aberrant differentiation of myeloid cells is accompanied by high numbers of circulating monocytes and granulocytes, whereas platelets and hemoglobin frequency decreases in the blood. This phenotype observed in mice is compatible with the side effects reported in humans after administration of therapeutic doses of IL-10 [31-34]. This study from our laboratory also demonstrated that EM was driven by IFNγ produced by T cells. However, the mechanism by which IL-10 reprograms T cells and induces IFNγ production is still unknown.

In this thesis, making using of the previously described pMT-10 mouse model [40], we sustained IL-10 over-expression for 30 days in order to characterized T cell alterations. Firstly, animals exposed to IL-10 presented an accentuated change in the ratio of CD4/CD8 T cells in the BM, led by the increase of CD4 T cells frequency in these animals as compared to control C57BL/6 mice. After IL-10 induction, pMT-10 mice also presented a high level of T cell activation, as indicated by the increased frequency of effector T cells ($CD44^+$ $CD62L^-$). In contrast, their naïve compartment ($CD44^-$ $CD62L^+$) was diminished. In addition, there was an accumulation of PD-1 and CD38 expressing T cells, not only in the BM but also in the spleen. This finding suggests that upon IL-10 exposure T cells transited to a highly activated state, which may be an indicator of T cell senescence [148]. We found that the T cell phenotype observed is also partly present in mice that over-express IL-10, but lack IFN-γ expression (pMT-10.IFNγ$^{-/-}$). In pMT-10.IFNγ$^{-/-}$ mice

there was an overall reduction of the naïve T cell compartment and expansion of the effector one. Strikingly, pMT-10.IFNγ$^{-/-}$ mice also presented an upregulation of PD1 expression in CD4 and CD8 T cells, demonstrating that the increase of PD1^{+} cells is independent of IFNγ signalling at least for the tested time point. As not all T cell alterations induced by IL-10 are lost in the absence of IFNγ, we suggest that the observed T cell phenotype is partly independent of the self-produced IFNγ. We are currently investigating the kinetics of the described T cell alterations, by repeating this experiment at earlier time points, such as 5, 10 and 15 days post-Zn administration in pMT10 and pMT-10.IFNγ$^{-/-}$ mice. In addition, to better understand to which extent the phenotype observed is independent of IFNγ expression, we will also analyse the transcriptome of CD4 and CD8 T cells purified from pMT-10 and pMT-10.IFNγ$^{-/-}$ mice exposed to IL-10.

After revealing that T cells exposed to IL-10 were in an activated state, we questioned if this strong and prolonged activation was altering T cell function. Knowing that T cells highly rely on healthy mitochondria to properly respond to stimulus and provide protection [108], we assessed mitochondrial functionality through specific dyes. In mice over-expressing IL-10 there was an increase in mitochondrial mass in BM and spleen CD4 T cells , which could indicate increased biogenesis due to its activated state [109, 110]. However, mitochondria enlargement after IL-10 induction is accompanied by decreased expression of MTR, indicating loss of mitochondrial fitness, which has been previously described in aged and exhausted T cells [142]. These results demonstrated that IL-10 induces mitochondrial dysfunction in T cells. This experiment will now be repeated to also include CD8 T cells in the analysis. In the future, we plan to investigate metabolic alterations in T cells after IL-10 induction through Seahorse and quantification of metabolic genes expression. In this note we will also obtain images of T cells through transmission electron microscopy, to better assess the extent of mitochondrial damage.

Mitochondrial dysfunction has been described in aged T cells [142]. Taking into consideration that T cells with dysfunctional mitochondria have been found to induce premature senescence and mitochondrial dysfunction is known to play a central role in cellular and organismal aging [131, 132, 176], we investigated senescent T cells in animals exposed to IL-10. Senescent T cells are commonly identified through the presence of DNA damage, being γH2AX a frequently used DNA damage marker [132]. Indeed, mice over-expressing IL-10 presented an increased γH2AX production by CD4 and CD8 T cells in the BM, indicating once again that T cells become dysfunctional after exposure to IL-10. In the future, we will also analyze the expression of CD153, another

marker of senescence in T cells [179] and the expression of SASP factors, as SASP presence directly relates to senescent cells and ageing [154].

The alterations found in T cells in mice over-expressing IL-10 are compatible with modifications induced by aging [131]. With this in mind and considering that the adipose tissue is one of the most studied tissues in the context of aging, its characterization after IL-10 induction was important to start unveiling the impact of IL-10 at the organism level. Firstly, through histologic analysis and quantification we found that mice exposed to IL-10 displayed a reduction in the adipocyte mean area. A previous report that characterized the adipose tissue of mice at different ages, found that while adipocyte mean size reaches its maximum in middle age, it decreases with advanced age [180]. It has been suggested that enlargement of adipocytes in middle age could be due to mitochondrial dysfunction [181], however it is not understood why this enlargement is followed by a reduction in size at a later age. In our study, we also found that the adipose tissue from mice exposed to IL-10 presented evidence of cellular infiltrates. In the adipose tissue, necrosis and apoptosis in known to induce infiltrations, generally of macrophages, which are designated as crown-like structures and are associated with inflammation and aged tissue [177]. We then decided to investigate possible alterations in the immune cell composition of the adipose tissue upon IL-10 exposure. Interestingly, in the IL-10-induced mice there was a decrease in the frequency of myeloid and B cells, which was accompanied by an increase in T cells frequency. It is important to mention that an increase of senescent T cells in the adipose tissue during aging has been previously described [163-165]. Thus, it is possible that the T cells that accumulate in the adipose tissue of induced pMT-10 mice are senescent. To test this hypothesis, we will now perform a more complete flow cytometry analysis of the T cells present in the adipose tissue, which will include surface markers such as PD-1, CD38 and CD153. We will also investigate whether alterations to the adipose tissue are lost in induced pMT-10.CD3$^{-/-}$ to fully assess the involvement of T cells to the observed phenotype. Finally, through a tissue staining with SA-β-gal, a common senescence marker [132] we demonstrated that IL-10 induction increased senescence in the adipose tissue. In the future we will also extract adipose tissue RNA to better understand IL-10 impact on the tissue, and we also plan to verify if other tissues such as the skin, kidney and liver present increased senescence after IL-10 induction

We then decided to evaluate the impact of IL-10 in the organism at a later time point. For this, we induced pMT-10 mice to over-express IL-10 for 75 days. It was noticeable that pMT-10 mice did not gain weight throughout time as it would be expected from healthy animals, and as seen in control C57BL/6 mice. Considering all the previously described

alterations in the adipose tissue, we questioned if this could be related to alterations in the quantity of adipose tissue. We quantified whole-body percentage of adipose tissue in pMT-10 and C57BL/6 mice after 75 days of IL-10 over-expression induction. Mice over-expressing IL-10 presented a lower body fat percentage mean, however this difference was not significant. Due to a lot of variability between the control animals (C57BL/6), it was not possible to fully address our question with the current data. We are soon going to increase the size of the experimental groups and assess SA-β-gal activity in the adipose tissue also at day 75.

Finally, the mechanism by which IL-10 reprograms T cells and induces IFNγ production is still unknown. To start gaining insights into this question we generated chimeric mice by transferring LSK cells isolated from the BM of IL-10Rα deficient pMT-10 mice (pMT-10.IL-10Rα$^{-/-}$) into sub lethally irradiated recipient pMT-10.CD3 deficient mice, generating mice where all T cells are unresponsive to IL-10. After reconstitution, mice were subjected to Zn administration for 10 days. The abolishment of the IL-10 receptor in T cells led to a decrease of IFNγ production by CD4 and CD8 T cells, when compared with pMT-10 mice. However, the IFNγ production by these cells seems to be higher in these chimeric mice when compared to control C57BL/6 mice. This likely suggests that IL-10-dependent and independent signals operate. To further confirm or not these results we will repeat this experiment with a higher number of mice per group. Chimeric mice had a high mortality rate after reconstitution, reason why we were unable to prolong the Zn administration for 30 days. In the future, we will repeat this experiment adding antibiotics to the mice diet, as we have confirmed that the presence of antibiotics in the diet does not alter the T cell phenotype after IL-10 induction (data not shown).

In summary, this work assigns novel and unexpected function to IL-10, particularly its ability to directly and/or indirectly inducing a profound reprogramming of CD4 and CD8 T cells towards a highly activated and cytotoxic state and likely a senescent phenotype. Furthermore, the data obtained so far suggest that these IL-10-reprogrammed T cells may infiltrate in the adipose tissue, causing premature senescence. Finding the broader effects of IL-10 and IL-10-reprogrammed T cells in the organism and the underlying mechanisms is an exciting avenue of research, with potential to improve IL-10 based therapies.

Concluding Remarks

We already knew that IL-10 induction *in vivo* reprogrammed CD4 and CD8 T cells, which led to production of IFNγ and consequently the development of EM. However, it was still not completely understood how IL-10 impacted T cells and its consequences on the organism. My thesis aimed at better comprehending these aspects and so far, our results suggest that IL-10 is able to alter CD4 and CD8 T cell phenotype, reprogramming T cells from a naïve to a cytotoxic state. It negatively impacts T cell function, leading to mitochondrial alterations. This will most likely result in deficient metabolism and overall cell dysfunction. Most importantly, IL-10 seems to act as a trigger for cellular senescence, having a strong impact in the organism, which we demonstrated through the adipose tissue, which is greatly involved in the organism homeostasis.

This work unveiled novel roles of IL-10, particularly is involvement in cellular alterations very similar to changes that occur during aging. In the future we hope to also validate the mechanisms trough which IL-10 acts to induce the described phenotype. This information can possibly contribute to the development of new IL-10 based therapies able to prevent such side-effects, playing a part in the treatment of inflammatory and autoimmune diseases. Of note, as mentioned above, due to mitochondrial dysfunction, metabolic dysfunctions will most likely also be present in these cells.

Overall, a more in-depth knowledge of IL-10 impact and the molecular pathways through which it acts will be helpful to further understand immune regulation during senescence and aging, while contributing to the development of new IL-10-based therapies.

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