Universidade do Minho
Escola de Ciências

Ana Catarina Pires Martins

Decoding the role of IL-10 in aging

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Abstract

Chronic inflammation is present in several diseases from autoimmunity to cancer, neurodegenerative diseases and others. A chronic state of low-grade inflammation, known as inflammaging, has also been linked to aging. To counteract this chronic inflammation, the anti-inflammatory cytokine interleukin (IL)-10 appears as a potential candidate. IL-10 has a broad role in regulating the immune system and has several distinct actions in different cells. Due to its great therapeutic potential in inflammatory and autoimmune diseases, understanding the impact of IL-10 in the organism homeostasis is fundamental. Our group has been investigating this issue, by resorting to a novel mouse model of inducible IL-10 over-expression (pMT-10). Previous results indicated that pMT-10 mice over-expressing IL-10 for 30 days develop a myeloproliferative phenotype, which is similar to that of animal models and patients with myeloproliferative neoplasms and is compatible with an aged hematopoietic system.

The main aim of this thesis was to extensively characterize at the organism level the effects of 75 days of sustained IL-10 over-expression. Interestingly, we found that pMT-10 mice die prematurely and present hair depigmentation, along with an histological phenotype suggestive of an aged skin. This skin phenotype is marked by a reduction of dermal and skin fat thickness, wound healing delay and alterations at the hair follicle (HF) level, including the frequency, length and melanin content. Resorting to an ex vivo model, we found the cellular phenotype of fibroblasts retrieved from pMT-10 mice that over-expressed IL-10 for 75 days to be compatible to that of elderly fibroblasts. Finally, using an in vitro system, we gained evidence that IL-10 triggers cellular senescence in mouse adult fibroblasts. Together, these data disclosed IL-10 over-expression to induce multiple phenotypes that parallel premature aging and age-related diseases. In all, this thesis brings novel insight on the biology of IL-10, which might shed light into novel targets involved in aging and into the importance of immune-driven aging, showing that immune balances, rather than inflammaging play a role in this process.

Keywords: Interleukin-10, Aging, Skin, Cellular senescence

Resumo

A inflamação crónica está presente em várias doenças desde autoimunidade, cancro, doenças neurodegenerativas, entre outras. O estado crónico de inflamação de baixo grau também está associado ao envelhecimento. Para contrariar esta inflamação, a citocina anti-inflamatória IL-10 aparece como um potencial candidato. A IL-10 tem variadas funções na regulação do sistema imune e atua de diferentes formas em diversas células. Devido ao seu potencial terapêutico em doenças inflamatórias e auto-imunes, é fundamental perceber o impacto da IL-10 na homeostasia do organismo. O nosso grupo tem estudado essa questão, utilizando para isso um modelo de murganho (pMT-10) na qual é possível induzir a expressão de IL-10. Estudos anteriores demonstraram que murganhos pMT-10 expostos à IL-10 por um período de 30 dias desenvolvem um fenótipo mieloproliferativo que se assemelha ao fenótipo apresentado por pacientes e modelos animais de neoplasias mieloproliferativas e, que para além disso, é compatível com um sistema hematopoiético envelhecido.

O principal objetivo desta tese é caracterizar extensivamente as alterações induzidas no organismo por 75 dias de expressão excessiva de IL-10. Nesse sentido descobrimos que murganhos pMT-10 expostos à IL-10 morrem prematuramente e desenvolvem despigmentação do pelo, acompanhado por um fenótipo histológico sugestivo de uma pele envelhecida. Este fenótipo é caracterizado por uma redução das camadas derme e hipoderme da pele, um atraso na cicatrização de feridas e alterações ao nível dos folículos capilares, incluindo a frequência, comprimento e conteúdo de melanina. Utilizando um modelo ex vivo, encontramos um fenótipo celular de fibroblastos retirados de murganhos pMT-10 que expressaram IL-10 por 75 dias semelhante ao de fibroblastos envelhecidos. Finalmente, usando um sistema in vitro, obtivemos evidências de que a IL-10 desencadeia senescência celular em fibroblastos de murganhos. No seu conjunto, estes resultados evidenciam múltiplos fenótipos induzidos pela expressão excessiva de IL-10 associados a um envelhecimento prematuro e a doenças relacionadas com o envelhecimento. Este trabalho revela assim novos aspetos da biologia da IL-10, fornecendo novos alvos envolvidos no envelhecimento precoce, e demonstra a importância de um sistema imunitário no envelhecimento.

Palavras-Chave: Interleucina-10, envelhecimento, pele, senescência celular.

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List of Abbreviations

BM	Bone marrow
CDK	Cyclin-dependent kinase
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
HF	Hair follicle
HSC	Hematopoietic stem cell
H&E	Hematoxylin and eosin
IL	Interleukin
IL-10R	IL-10 receptor
MAF	Murine adult fibroblast
MAPK	Mitogen-activated protein kinase
MiDAS	Mitochondrial dysfunction associated-senescence
NEB	Nuclear envelope breakdown
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
rIL-10	Recombinant IL-10
RT	Room temperature
SASP	Senescence-associated secretory phenotype
SA-βGal	Senescence-associated β-galactosidase
SC	Stem cell
STAT3	Signal transducer and activator of transcription 3
TNF	Tumor necrosis factor
Zn	Zinc

1. INTRODUCTION

1.1 Aging: general concepts

Aging is an inexorable fate of biological organisms. It is broadly defined as a progressive, time-dependent loss of physiological functions of multiple cells and tissues of the organism. This deterioration results in a high vulnerability to environmental challenges, leading to aging-associated pathologies that sooner or later end up causing death[1]. However, the fundamental mechanisms that drive aging are not clearly understood, representing an obstacle in the development of interventions to attain healthy aging and longevity.

Organismal aging occurs with the concomitant aging of its systems. The immune system is no exception, showing a series of age-related alterations, globally termed immunosenescence. These alterations include: deteriorations on the production of immune cells and on the capacity to efficiently respond to infectious agents; reduced effectiveness of vaccination; and enhanced susceptibility to autoimmune diseases and cancer, such as leukemia[2,3]. Importantly, immunosenescence is among the aging-associated factors with a greater negative impact on the quality of life of the elderly[2,4]. It is now accepted that immunosenescence results from a series of alterations that are imposed to the hematopoietic process and reflected in the mature immune cells populations. The next sections will dwell on the cellular and molecular mechanisms associated with aging and on the problematic of immunosenescence.

1.2 Cellular senescence in aging

During their lifetime, cells are continually exposed to stress and damage from endogenous and exogenous sources, to which they respond attempting complete recovery or death. Proliferating cells can initiate an additional response to prevent propagation of damaged cells by adopting a permanent and irreversible state of growth arrest that is referred to as cellular senescence[5–8]. In addition to being a potent tumor suppressor mechanism[9–11], the cellular senescence response has been implicated in tissue remodeling that acts during normal embryonic development[12,13] and upon tissue damage[14,15], as well as in organismal aging[16,17]. Contrasting with these beneficial roles of cellular senescence, there is mounting evidence involving this process as an important driver of aging and multiple age-related disorders, including pulmonary fibrosis, atherosclerosis, osteoarthritis and Alzheimer´s disease[17,18].

Senescent cells accumulate in some, but not all, organs and tissues over time and are present at sites of age-associated pathology[19,20]. The most significant increases are observed in the lung, spleen, liver and skin[21]. It is not known whether this rise reflects an increased rate of generation, decreased rate of clearance or both. An explanation for the duality in the role of cellular senescence in complex organisms is that it may be the consequence of evolutionary antagonistically pleiotropic gene action – that is, genes or processes that ensure early-life survival will be selected even if they promote late-life disease, such as aging phenotypes[22]. This is a key concept to understand many aspects of aging, particularly the link between aging and cancer[23]. Indeed, cellular senescence balances the benefits of preventing the proliferation of potentially damaged cells in young age against the drawbacks of rid tissues from functional cells needed to maintain homeostasis, contributing to age-related pathologies (Figure 1)[24,25].

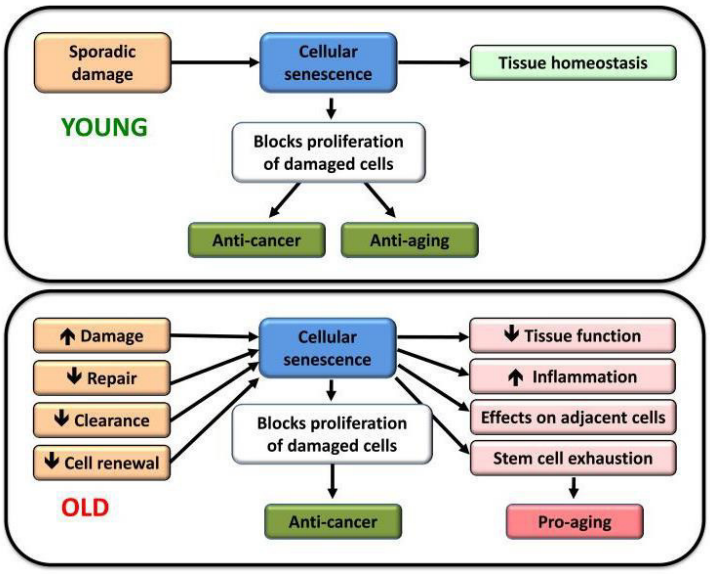

Figure 1. Cellular senescence contributes to aging. In young organisms, cellular senescence blocks propagation of damaged and potentially malignant cells from the tissues, thus protecting from cancer and contributing to tissue homeostasis. To accomplish this purpose, an efficient cell replacement system that culminates in the demise of senescent cells and mobilization of progenitors to re-establish cell number is required. In old organisms, this process may become ineffective or may exhaust the progenitor stem cells regenerative potential, which may further be aggravated by excessive senescence-associated secretory phenotype (SASP), eventually leading to the accumulation of senescent cells. This combination of factors gives rise to a pervasive damage and contributes to aging. From López-Otín et al.[1]

Among the plethora of stimuli that elicit cellular senescence are: telomere attrition resulting from consecutive cell division (termed replicative senescence), severe or irreparable DNA damage, oxidative stress, chromatic perturbations (genotoxic stress), mitochondrial dysfunction and expression of certain oncogenes (oncogene-induced senescence)[26,27]. These stressors are signaled through various intracellular pathways, many of which activate the p53

tumor suppressor protein, and almost all of them converging to the transcriptional activation of the cyclin-dependent kinase (CDK) inhibitors p15 (encoded by CDKN2B; also known as INK4B), p16 (encoded by CDKN2A; also known as INK4A), p21 (encoded by CDKN1A; also known as WAF1) and p27 (encoded by CDKN1B) (Figure 2)[17,28]. In turn, the inhibition of CDK-cyclin complexes drives entry into proliferative arrest by preventing retinoblastoma phosphorylation and inactivation, and subsequent cell cycle progression[29]. In addition to several stressors (with different severities) and signaling networks, the propensity with which cells engage one or other pathway and the mechanisms that ultimately induce senescence may also vary, depending on the conditions and cell type[24].

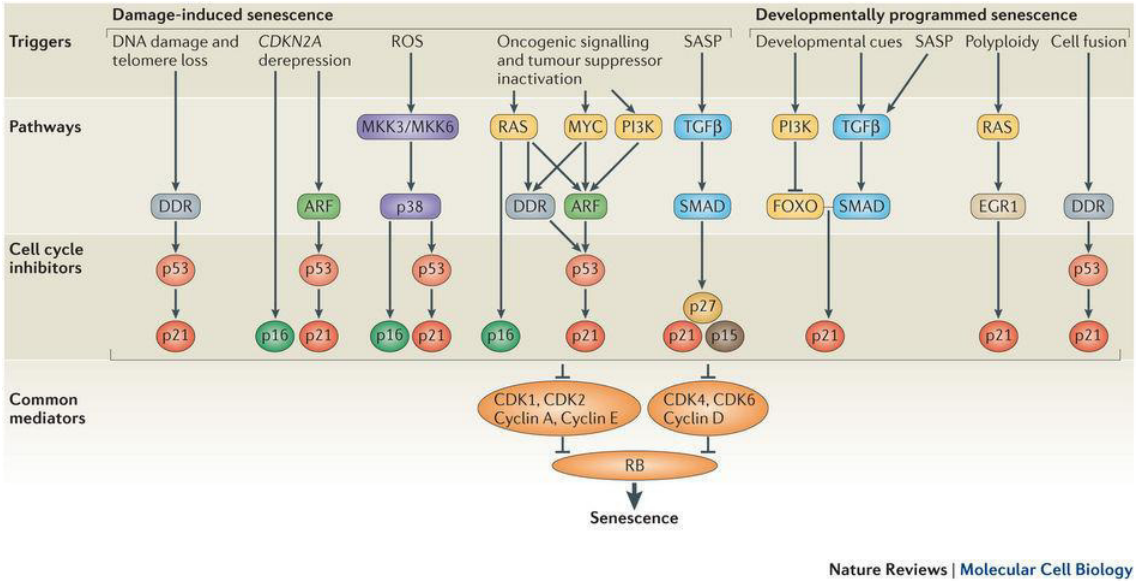

Figure 2. Cellular senescence program. A variety of stimuli (triggers) engage cellular signaling cascades (pathways) that ultimately activate cell cycle inhibitors and the tumor suppressor retinoblastoma (RB). DNA damage agents and telomere loss phosphorylate and activate p53 and its downstream transcriptional target p21, mediated by DNA damage response (DDR). Many types of senescence are linked to the derepression of the cyclin-dependent kinase inhibitor 2A (CDKN2A) locus, which encodes two important tumor suppressors, p16 and the p53 activator ARF. Reactive oxygen species (ROS) activate p16 and p53 through the kinase effector p38. Oncogenic signaling and tumor suppressor loss upregulate p16 and increases the transcriptional activity of p53, the latter being mediated by DDR and ARF. Transforming growth factor-β (TGFβ), a key component of the senescence-associated secretory phenotype (SASP), induces the activation of the cell cycle inhibitors p15, p21 and p27 through the SMAD complex. Development cues induce the expression of p21, which is controlled by the phosphoinositide 3-kinase (PI3K)-forkhead box protein O (FOXO) and TGFβ-SMAD pathways. Polyploidy and cell fusion also upregulate p21 through the RAS-induced activation of the early growth response protein 1 (EGR1) and DDR-induced p53, respectively. From Muñoz-Espín et al.[28]

Cellular senescence may impact on aging through two distinct mechanisms. First, cellular senescence might gradually limit the regenerative ability of tissues by exhausting the supply of progenitor and stem cells (SCs)[30,31]. Second, the senescent phenotype frequently results in secretion of degradative enzymes, inflammatory cytokines and growth factors that

might disrupt the normal tissue structure and function[31–33]. Interestingly, interventions to selectively eliminate senescent cells from a progeroid model seem to have a positive impact by preventing or delaying age-related deterioration and attenuating the progression of already established pathologies[34,35]. Thus, therapeutic strategies that could specifically target senescent cells are an emerging promise to rejuvenate tissues and maximize healthy lifespan[34,36–39].

1.2.1 Identification of senescent cells

Owing to the multiple implications of senescent cells in the normal function of the organism and to their involvement in its dysfunction, the detailed study of senescent cells, namely their identification and characterization, has gained importance over the years.

Senescent cells contrast with other non-dividing cells (such as quiescent or terminally differentiated cells) by morphological changes and the expression of several markers. In vitro, senescent cells generally show an increased size, suggesting that the macromolecule synthesis continues without cell division, become flat, vacuolized, occasionally, multinucleated and exhibit enlarged and irregular nuclei. However, because of the tissue architecture, in vivo senescent cells retain their normal morphology[7,28,40]. Senescent cells display other characteristics that collectively allow their identification both in vivo and in vitro, including the senescence-associated β-galactosidase (SA-βGal) activity based on increased lysosomal content[41], the absence of proliferative markers, the expression of cell cycle inhibitors, tumor suppressors and DNA damage markers, nuclear foci of heterochromatin and signaling molecules secretion[7,28,40,42].

Moreover, senescent cells remain metabolically active, but undergo widespread phenotypic changes, including resistance to apoptosis[43] and profound chromatin organization and gene expression pattern alterations, ultimately developing a complex pro-inflammatory response known as senescence-associated secretory phenotype (SASP)[44,45]. The SASP includes the secretion of multiple inflammatory cytokines (e.g. interleukin (IL)-1, IL-6 and IL-8) and chemokines [e.g. chemokine (C-X-C motif) ligand 1 (CXCL-1), -3 and -10], growth factors [e.g. transforming growth factor-β (TGFβ), hepatocyte growth factor and granulocyte/macrophage colony-stimulating factor (GM-CSF)] and matrix-remodeling proteases [e.g. matrix

metalloproteases] that can influence the neighboring cells by activating cell-surface receptors and corresponding signal transduction cascades[46,47].

Importantly, none of these markers is on its own entirely exclusive or universal for the senescent state and for all senescent types, making the identification of senescent cells challenging, especially in vivo. Therefore there is ample consensus on the relevance of using a combination of features and markers to identify these cells[42]. The cells of the immune system also age, a topic that I will develop in the next sections.

1.3 The aging of the hematopoietic system

Hematopoiesis is a continuous and regulated process that ensures differentiation of hematopoietic stem cells (HSCs) into mature blood cells, functioning mainly in homeostasis and during immune responses[48]. HSCs are endowed with two unique properties, the ability to self-renew to preserve the SC pool, and to differentiate to give rise to all terminally differentiated cells through a cascade of myeloid and lymphoid progenitor cells[49,50]. HSCs reside as rare cells within the bone marrow (BM), which presents a specialized microenvironment - termed the HSC niche – that provides soluble factors and cell-cell interactions that directs HSC fate and function[51,52,53]. Both an endosteal and a vascular niche have been identified for HSCs[54].

Current data support that aging of the immune system is initiated at the very top of the hematopoietic hierarchy and that aging of HSCs directly correlates with immunosenescence[55,56,57]. Advancing age is accompanied by increased number of HSCs in the BM[58–61]. However, this increase does not translate into improved HSC function, as several functional deficiencies in aged HSCs of murine models have been described[62–64]. As determined in serial transplantation studies, aged HSCs show increased long-term self-renewal activity (Figure 3)[60,61], and overall reduced reconstitution potential that may be primarily driven by age-related accumulation of DNA damage[65–67]. Furthermore, when young and aged HSCs are transplanted together into sub-lethally irradiated young recipients, aged HSCs are less efficient in contributing to hematopoiesis compared to young HSCs and possess a lower ability to home to the BM[68,69]. Moreover, as compared to young HSCs, aged HSCs localize more distantly to the endosteum and present significantly higher cell protrusion activity in vivo. This correlates with weakened ability to

adhere to stromal/niche cells as well as reduced polarity upon adhesion, suggesting altered, less favorable interactions of aged HSCs with the niche, and thus altered function[59,70]. Indeed, the HSC mobilization ability from the BM to peripheral blood, which requires de-adhesion from the niche, is enhanced in old HSCs[71].

In addition to changes in self-renewal and reconstitution potential, aging has been implicated in skewing the capacity of HSCs to differentiate towards myeloid lineage versus lymphoid lineage[56]. Concomitantly, a number of events taking place downstream of HSC specification, such as thymic involution, might also contribute to this phenotype[72]. Compared to young HSCs, the hematopoietic differentiation of old HSCs exhibits a markedly reduced lymphoid and erythroid production, specifically decreased number of common lymphoid progenitor cells combined with relative loss of B-lymphoid progeny[58,73]. However, because the myeloid lineage potential is maintained or even increased a marked enhancement of myeloid cells is observed[68,74]. Consistent with this, gene expression profiling of purified HSCs from young and old mice identified mostly up-regulation of myeloid genes and down-regulation of lymphoid genes[60]. Similar tendencies have been observed in the human hematopoietic system. This aging-associated lineage skewing can be explained as the outcome of gradual alterations in the clonal composition of the pool of HSCs. Such a view is supported by data revealing that myeloid-biased HSCs accumulate in the old HSC pool at the expense of lymphoid-bias or balanced HSCs[75–77]. Clonal assays on sorted young and aged HSCs though showed that myeloid-dominant HSCs, from aged BM also exhibit a delayed proliferation response, a reduced BM homing in vivo, and additional functional defects, implying that all the HSCs progeny age[78]. Myeloid dominance may also have implications in age-related hematopoietic malignancies, such as the increased predominance of myeloid leukemias in the elder population[56]. Taken together, these observations point to a combination of both cell-intrinsic mechanisms, that is, changes in individual HSCs and changes in the composition of the HSC pool, as causes of aging-induced hematopoietic lineage skewing[55].

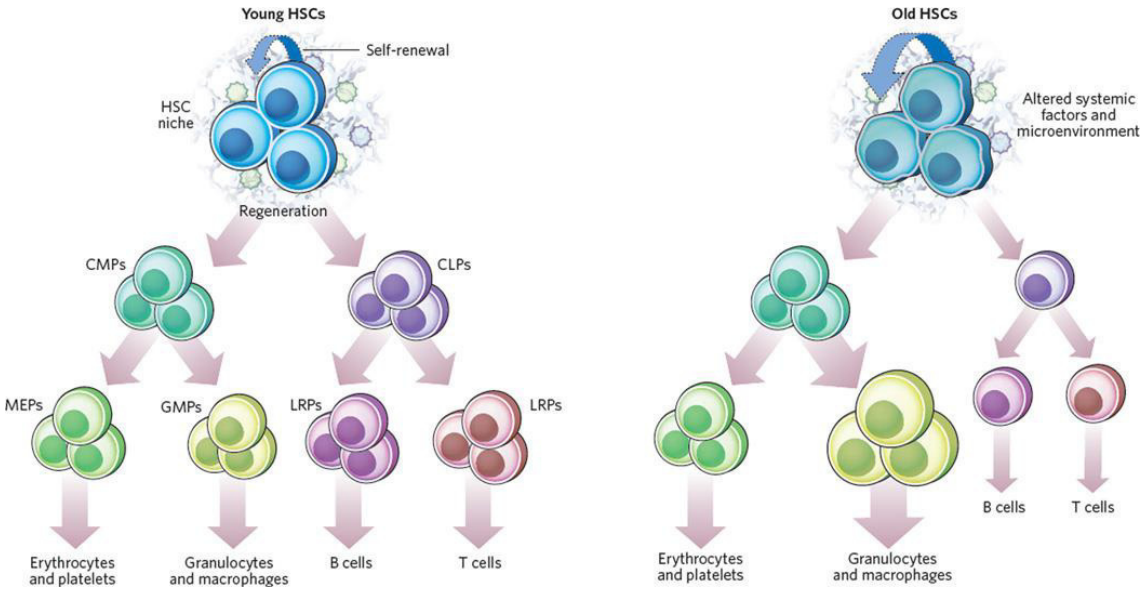

Figure 3. Functional decline in HSCs with aging. Major functional differences between young (left) and old (right) hematopoietic stem cells (HSCs) that occur with age are shown, particularly increased stem cell self-renewal, reduced regenerative capacity and differential capacity to generate lymphoid and myeloid progenitors. Aged HSCs exhibit attenuated output of common lymphoid progenitors (CLPs), and consequently B and T cells (immunosenescence), whereas common myeloid progenitors (CMPs) are generated at the same rate as by young HSCs. In turn, the number of common granulocyte-macrophage progenitors (GMPs), and consequently granulocytes and macrophages increases upon aging, albeit megakaryocyte-erythrocyte progenitors (MEPs) lineage is not altered. Cell-extrinsic factors that possibly drive or exacerbate HSCs aging include niche-secreted and systemic factors. LRPs, lineage restricted progenitors. From Ergün Sahin et al.[79]

At the molecular level, age-dependent alterations in the biology of HSCs might also be governed by the acquisition of defects in telomeres, mitochondrial DNA or genomic DNA[56,80]. Studies in Terc$^{-/-}$ mice have shown that telomere dysfunction activates cell-intrinsic checkpoints that limits the proliferative capacity of HSCs, as well as provokes environmental defects that can impair the function and engraftment of HSCs[81,82]. The dysfunctional environment induced by telomere dysfunction affects B lymphopoiesis and increases myeloid proliferation[83]. However, reports on telomerase (TERT) transgenic mice have shown that there is also a telomere-independent barrier reducing the repopulating potential of HSCs[84]. One possible mechanism is the accumulation of extra-telomeric DNA damage imposed by transplantation-mediated stress[67,85]. Recent publications also show increased expression of the tumor suppressor and cell cycle regulator p16 in aged HSCs, and this at least in part might contribute to the functional decline of these populations with age[86]. p16 up-regulation is also associated with cellular senescence, thus indicating that it might in general be activated in response to DNA damage. In addition to DNA damage, activation of the p38 mitogen-activated protein kinase (MAPK) in response to increasing levels of reactive oxygen species (ROS) contributed to exhaustion of SC population during serial

transplantation, therefore implying a role of ROS-p38 MAPK pathway in limiting HSCs self-renewal[82].

The aging of HSCs in addition of being partly stem cell intrinsic, is also extrinsically regulated by the local (niche) and the systemic environment, including cytokines, hormones and neuropeptides[56,87]. Changes in the HSC niche composition and function with age involve reduced bone formation, altered composition of extracellular matrix and increased adipogenesis. The aging BM microenvironment of HSCs is also likely a critical player in shaping the HSC clonal compartment over time. Some studies have shown that an aged microenvironment might promote myelopoiesis over lymphopoiesis, and might have a negative influence on HSC function[57,85]. There is also evidence that the aged niche contributes to the expansion of the pre-leukemic HSCs number in the context of myeloproliferative diseases[88].

All these alterations in the aging hematopoietic system are then reflected in an increased incidence of myeloproliferative diseases, including acute myeloid leukemia[89,90], decreased competence of both the innate and adaptive immune system[72,91], and increased propensity for anemia[92,93]. They are also reflected in a deterioration of the innate [monocytes, dendritic cells (DCs) and natural killer cells] and adaptive immunities (B and T lymphocytes), which combine to cause a substantial reduction of immune responsiveness in the elderly. However, the lymphoid compartment is generally more affected than the myeloid one[4].

1.4 Chronic inflammation in aging

Aging is associated with a pro-inflammatory environment characterized by dysregulation in cytokine secretion. High plasma concentrations of IL-1β, IL-6 and tumor necrosis factor (TNF) have been described in elderly people and are considered powerful predictive markers of morbidity and mortality[94,95]. These factors contribute to a lifelong continuous stimulation of the immune system, resulting in a chronic, subclinical state of low-grade inflammation, named "inflammaging", that may augment tissue damage from infections in old individuals[96,97]. Inflammaging is a highly risk factor for the emergence and progression of most if not all age-related diseases, including osteoporosis and neurodegenerative diseases[98]. Thus, immunosenescence in the innate immune system is involved in the reduced ability to respond to

pathogens and in the impaired capacity to collaborate in the initiation of the adaptive immune response[99].

SASP is considered the main contributor of inflammaging[98]. Globally, it constitutes a mechanism allowing damaged cells to communicate their compromised states and modulate the tissue microenvironment. The SASP represents one of the darkest sides of the senescence response, but it can also have positive effects, depending on the physiological context and age of the organism. SASP can spread senescence to surrounding cells in a paracrine manner, through a mechanism that generates ROS and DNA damage[44,100]. On the other hand, the SASP factors can function in an autocrine manner to reinforce the senescent state through a positive feedback loop that maintains their expression, which is beneficial to reduce the risk of oncogenic transformation[33]. SASP helps to recruit immune cells for tissue repair, thereby creating an inflammatory microenvironment that promote the elimination of the damaged cells[33,101]. SASP has also been shown to promote cancer cell growth and invasion[9,101]. Further, the proinflammatory factors of the SASP are thought to cause chronic inflammation, which in turn is associated with aging and development of age-related diseases[102]. Although the SASP is senescence-associated, it does not appear to be a consequence of either p53/p21 or p16-dependent growth arrest[103]. Rather, it is known to be positively regulated by persistent DNA damage response signaling, by proteins that act upstream p53[104], and/or p38 MAPK cascade[105], depending on the context and nature of the senescence-inducing stimuli.

1.5 IL-10: an anti-inflammatory cytokine with pleiotropic effects in the immune response

An effective immune response faces the challenge of fighting insults, such as invading pathogens and tissue damage, whilst avoiding injury to the host. It is not surprising, therefore, that the immune system has evolved parallel mechanisms to counteract and usually eliminate the immunopathology driven by excessive immune activation. One of the foremost regulatory mechanisms is the production of the anti-inflammatory cytokine IL-10, that strikes the immune system balance between pathology and protection[106,107].

To exert its actions, IL-10 homodimers bind to a tetrameric transmembrane receptor that comprises two IL-10 receptor (IL-10R) 1 and two accessory IL-10R2 molecules[106]. IL-10R1 specifically binds IL-10 and induces a conformational change in the cytokine that creates a binding site for IL-10R2, enabling the oligomerization of IL-10R2 and assembly of the complex[108]. IL-10R2 alone is unable to bind IL-10. IL-10R2 has also an important role in the recruitment of the downstream signaling pathways[106]. Upon IL-10 binding, the IL-10R activates two members of the Janus kinase family, Janus kinase 1 and tyrosine kinase 2, which then phosphorylates IL-10R1 on two tyrosine residues, thereby creating docking sites for the signal transducer and activator of transcription 3 (STAT3)[109]. The signaling cascades initiated ultimately lead to the dimerization and nuclear translocation of STAT3 that allows the expression of target genes with important roles in dampening the immune response[110].

IL-10 has emerged as a regulator of infection, with a key role in preventing excessive immunopathology[111]. The genetic ablation of IL-10 is often responsible for enhanced resistance of the mice to a variety of intracellular pathogens, including *Toxoplasma gondii*, *Trypanosoma cruzi*, *Candida albicans*, *Listeria monocytogenes*, *Mycobacterium avium*, *Mycobacterium bovis* and *Plasmodium chabaudi*[106,112,113]. However, this increased pathogen clearance often comes at the expense of severe tissue pathology[111]. This is not always the case as during other infections, IL-10 deficiency results in a better elimination of the pathogen without causing damage to the host[114,115].

IL-10 and IL-10R signaling are also major regulators of intestinal immune homeostasis in mice and humans[116,117]. Loss-of-function mutations in both IL-10 and IL-10R are strongly associated with early onset of inflammatory bowel disease[118–120]. In the mouse model, IL-10 inhibition results in spontaneous colitis in the presence of normal gut flora and recent studies have shown that macrophage-restricted IL-10R deficiency also cause a spontaneous colitis profile[121–123]. Additionally, increasing evidence demonstrates that IL-10 plays a central role in the onset and development of autoimmune diseases, such as psoriasis, multiple sclerosis or rheumatoid arthritis[106,124,125].

The major suppressive function of IL-10 involves the inhibition of pro-inflammatory cytokines production such as TNF-α, IL-1, IL-6, and IL-12, through its action on macrophages and DCs, and downregulation of the expression of major histocompatibility complex (MHC) class II antigens and co-stimulatory molecules (Figure 4)[106,126]. Moreover, this cytokine limits the

development of T helper 1-cell responses, by the inhibition of IL-12 secretion by antigen presenting cells, and also affects T helper 2-cell and allergic responses[127]. IL-10 also prevents the generation of DCs from monocyte precursors and blocks the ability to kill intracellular organisms by macrophages, in part by restricting their TNF production[107,128]. The mechanisms responsible for the inhibitory properties of IL-10 are to a large extent mediated by STAT3 signaling, downstream to the IL-10R[128].

Interestingly, in addition to its suppressive effects, IL-10 has been described to exert positive effects in other immune cells, including enhancement of B cell responses, by upregulating the MHC class II expression, $CD8^+$ T cells and natural killer cells functions. In some situations, IL-10 can also stimulate the expression of specific genes in toll-like receptors-activated phagocytes, induce the differentiation of IL-10-secreting $CD4^+$ regulatory T cells and promote the migration and differentiation of cytotoxic T lymphocytes[107,129]. The overall functions of IL-10 are depicted in Figure 4.

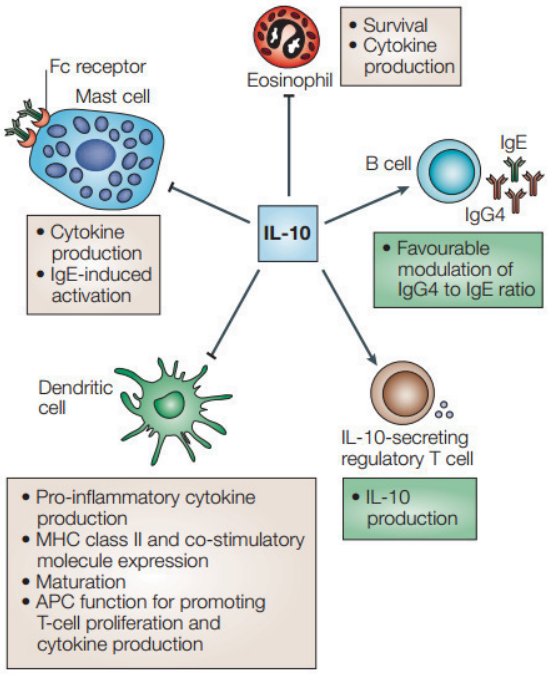

Figure 4. Actions of IL-10 in different cells. Interleukin-10 (IL-10) is a cytokine with multiple, pleiotropic effects in the immune system. IL-10 limits cytokine generation by mast cells and eosinophils, and inhibits the activation of mast cells as well as survival of eosinophils. IL-10 acts on dendritic cells, inhibiting antigen-presenting cell (APC) function and inhibiting the expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules. It also inhibits maturation of these cells. The stimulatory effects of IL-10 involve the increase of immunoglobin isotype switching in B cells and the induction of IL-10-secreting regulatory T cells. From Hawrylowicz CM et al.[127]

There are other possible, much less known effects of IL-10, one of which is in aging. In fact, there is an early suggestion that a dysregulation of IL-10 production modulated immunosenescence[130]. Other studies suggest a positive association between high IL-10 production and longevity, together with the reduced risk to age-related diseases, such as cardiovascular and inflammatory pathologies[131,132]. However, discordant results obtained in genetic association studies failed to assign a solid correlation between the presence of polymorphisms in the IL-10 gene locus and the longevity of the individuals[94,133–138]. Thus, a final conclusion as to whether IL-10 participates in the aging process has not been reached, which calls for deeper studies in this field.

1.5.1 A novel mouse model of inducible IL-10 over-expression: pMT-10 mice

As mentioned before, IL-10 has been intensively investigated in the last decades due to its involvement in preventing inflammatory and autoimmune diseases. The therapeutic potential of IL-10 has led to innumerable studies in mouse models and to a variety of clinical studies to treat patients with inflammatory diseases such as psoriasis, Crohn´s disease, or rheumatoid arthritis[124]. The perspective of a wider use of immune therapies based on IL-10 calls for the detailed understanding of the consequences of elevated IL-10 on the organism homeostasis. However, potential side effects to the exposure of the organism to this cytokine have not been yet established.

To this end, our group is exploring a novel and unique mouse model of inducible IL-10 over-expression, the pMT-10 mouse. This is an excellent tool for studying the impact of elevated IL-10 levels in the organism homeostasis, as it allows for the timely control of IL-10 expression in the absence of other immune deregulations[139,140]. These mice express IL-10 under the control of the zinc (Zn)-inducible sheep metallothionein promoter (Figure 6A). As a result, the levels of IL-10 sharply increase upon Zn administration and remain high, unless the Zn administration is suspended (Figure 6B). Transcriptomic analysis of different organs and cellular compartments of induced pMT-10 mice identified skin, BM and small intestine $CD45^-$ $TER119^-$ subsets as the main producers of IL-10 (Figure 6C). Thus, the pMT-10 mouse model allows for timely controlled IL-10

over-expression in specific anatomic locations, accompanied by an increase of this cytokine in the blood.

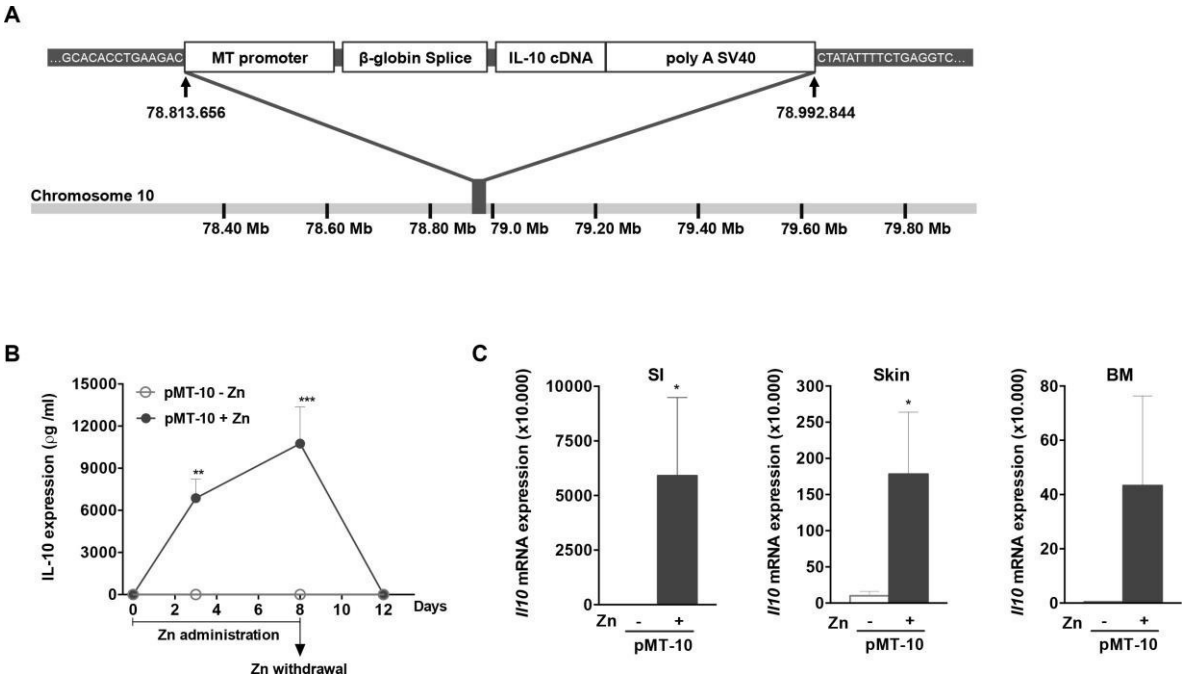

Figure 5. A novel mouse model for inducible IL-10 over-expression: pMT-10 mice. (**A**) Schematic representation showing the targeting vector and insertion site. (**B**) Kinetics of IL-10 over-expression in the serum at different time-points after zinc (Zn) administration and Zn suspension. pMT-10 mice were fed with normal (pMT-10-Zn) or Zn-enriched water (pMT-10+Zn) and at the indicated time-points blood was collected and the amount of IL-10 in the serum measured by immunoassay. (**C**) qRT-PCR identified $CD45^-TER119^-$ cell subsets from small intestine (SI), skin and BM as the main producers of IL-10 in pMT-10 mice fed with Zn-enriched water for 8 days. In both (**B**) and (**C**), each point or bar represents mean ± SD for 3 independent mice. Statistical differences were assessed by (**B**) two-way ANOVA (Sidak's multiple comparisons test) or (**C**) student's t test, $*p<0.05$, $**p<0.01$, $***p<0.001$. From Ana Cardoso et al.[140]

Recently, the use of this model contributed to better explore the mechanisms of the immune regulation elicited by IL-10 in the context of intestinal inflammation[140]. Moreover, the detailed study of this mouse model has so far disclosed an abnormal hematopoietic phenotype dependent on IL-10 over-expression (Ana Cardoso et al. unpublished). After IL-10 over-expression for 15 days, pMT-10 mice developed a myeloproliferative phenotype characterized by an increase in granulocyte-macrophage progenitors, accompanied by reduced B lymphopoiesis and anemia. This augmented myelopoiesis was accompanied by a marked splenomegaly. This phenotype parallels that of human and mouse models with myeloproliferative neoplasms[141–145]. This impact of IL-10 in hematopoiesis affects the BM, requires the expression of IL-10R in myeloid cells and starts as early as day 7 post-IL-10 over-expression, being fully established on day 30. In this

thesis, I continued to explore the pMT-10 mouse model, with the global aim of understanding the long-term effects of IL-10 over-expression in the organism.

2. RESEARCH OBJECTIVES

Given the therapeutic potential of IL-10 in many diseases, from auto-immunity and cancer, to neurodegenerative and inflammatory pathologies, understanding its impact in the organism homeostasis is fundamental. The aberrant myeloproliferative phenotype developed by pMT-10 mice after IL-10 exposure for a short period, led us to question which alterations are imposed to the organism homeostasis upon sustained long-term IL-10 exposure. By further exploiting the pMT-10 mouse model, this thesis proposes to gain additional insight on the effects of this cytokine at the organism level. The specific research objectives were:

1. To fully characterize the extent of the IL-10 over-expression in organism homeostasis;
2. To gain mechanistic insights on the alterations imposed by IL-10 to the organism homeostasis, by setting up the appropriate (in vivo, ex vivo and in vitro) systems.

In all, this work is relevant since it holds the potential of unveiling novel and unexpected aspects of the IL-10 biology, which is important to alert for potential risks associated with IL-10 administration as an immune therapy. Furthermore, it will open new avenues of research raising new questions and offering new experimental models.

3. MATERIAL AND METHODS

3.1 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). Mice were euthanized by CO_2 inhalation with efforts to minimize suffering.

3.2 Animals

The study described involved the use of the following 8-14 weeks-old-male mice: wild-type C57BL/6, pMT-10 - IL-10 inducible mice[139], and pMT-10 crossed with IL-10Rα deficient mice (pMT-10.IL-10Rα$^{-/-}$)[146]. The promoter driving IL-10 over-expression in pMT-10 mice is a metallothionein-induced promoter, which is activated in the presence of 50 mM of Zn in the organism, administered in the drinking water. IL-10 over-expression was induced by feeding the animals with a solution of 50 mM Zn sulphate heptahydrate (Sigma-Aldrich, MO, USA) and 2% sucrose (PanReac AppliChem, Germany) in the drinking water. Control animals were fed with 2% sucrose in the drinking water. Food was *ad libitum* for all animals. All the animals were bred and maintained at the Instituto de Investigação e Inovação em Saúde (I3S), except the pMT-10.IL-10Rα$^{-/-}$ which were bred at the Pasteur Institute (France).

3.3 Wound healing assay

Mice were anesthetized using isoflurane (5% induction and 2.5% maintenance). Following hair removal from the back area, two dorsal 3 mm wounds were created, in either side of the midline, using a biopsy punch. Every 2 days for a period of 8 days, the mice were anesthetized in the same conditions and the wounds measured along the x, y and z-axes using a digital caliper. The wound area was calculated from the average of three diameter measurements along the x, y and z-axes. Wound closure is expressed as a percentage of initial wound area at day 0.

3.4 Organ harvesting

To collect the organs to perform the desired experimental assays, mice were euthanized by CO_2 inhalation. After euthanasia, whole blood was collected by cardiac puncture and used to collect serum, by performing a first centrifugation at 5000 rpm for 2 minutes, and a second centrifugation of the supernatant at 10000 rpm for 10 minutes. The skin, eyes, spleen, kidney and small intestine were collected for histological analysis.

3.5 Histology

For histopathology, the harvested tissues were fixed in 10% formalin (Merck, Germany) for 48 hours at room temperature (RT) and were then embedded in paraffin. Paraffin-embedded tissue specimens were cut into 3-mm-thick sections (Rotary Microtome HM335E, MICROM International GmbH, Walldorf, Germany). Sections were then deparaffinised, rehydrated and stained with hematoxylin and eosin (H&E) (Gibco, Thermo Fisher, CA, USA), according to standard technique. Dorsal skin sections were also stained with Masson Fontana (Melanin stain). All images were acquired on an optical microscope using a DP 25 Camera and Software Cell B (Olympus, NY, USA). H&E quantifications of skin thickness and hair follicle (HF) length were performed using Cell B software. Both skin thickness and HF length were determined by taking 5 random measurements along the length of individual skin samples from telogen and anagen stage, respectively. The distance between two adjacent HFs was measured, and HF frequency was calculated as the number of HFs per mm of telogen stage skin section. At least five randomized different areas in each sample were analysed.

3.6 Enzyme-linked immunosorbent assay (ELISA)

IL-10 cytokine level in the serum was determined using eBioscience Ready Set-Go® kits, according to manufacturer's instructions.

3.7 Isolation and culture of mouse fibroblasts

For phase contrast live cell imaging, murine adult fibroblasts (MAFs) were collected from ears of 8-14 weeks-old C57Bl/6 and pMT-10 male mice fed with Zn for 75 days. Ears were washed with phosphate-buffered saline (PBS), cut into small pieces, and incubated with 1 mg/mL collagenase D (both from Roche Applied Science, Germany) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with nutrient mixture F-12 (Gibco, Thermo Fisher Scientific, CA, USA) for 45 minutes in a humidified atmosphere with 5% CO_2 at 37°C. Cells were then grown on a 6-well dish containing DMEM:F12, supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic-antimycotic (both from Gibco, Thermo Fisher Scientific, CA, USA).

For immunostaining, MAFs cultures were established from ears of 6 months-old C57Bl/6 mice. Cells were grown on a 6-well dish containing DMEM:F12, supplemented with 10% FBS and antibiotic-antimycotic. Cells that grew from tissue fragments were transferred to 25-cm^2 flasks and cultured to 90% confluency. The cells were then trypsinized and expanded into a 75-cm^2 flask. From this enriched MAF population, 1 x 10^4 cells were seeded in 24-well dish on sterilized 13 mm round glass coverslips (VWR, PA, USA) coated with 50 µg/ml fibronectin (Sigma-Aldrich, MO, USA). Cells were then incubated for 24 hours, 3 and 7 days in medium containing 50 ng/ml of recombinant IL-10 (rIL-10) (R&D Systems, UK). In the cultures incubated for 7 days the stimulation with 50 µg/ml of rIL-10 was repeated 4 days after incubation.

3.8 Immunostaining

For analysis of 53BP1 and Cdkn1a/p21 biomarkers, cells were quickly rinsed in PBS, fixed in freshly prepared 2% paraformaldehyde (2% PFA) (Delta Microscopies) in PBS for 20 minutes at RT, washed 3 times with PBS and permeabilized in PBS + 0.3% Triton-X100 (Sigma-Aldrich, MO, USA) for 7 minutes at RT. Cells were then washed with PBS-T [PBS with 0.05% Tween 20 (Sigma-Aldrich, MO, USA)] and blocked in 10% FBS in PBS-T for 1 hour at RT. Cells were incubated overnight at 4°C with primary antibodies diluted in PBS-T + 5% FBS. Then, cells were washed with PBS-T, and incubated with secondary antibodies diluted in PBS-T + 5% FBS at RT for 45 minutes. Cells were finally washed with PBS-T, stained with HCS CellMask™

(Invitrogen, CA, USA) and counterstained with 1 µg/ml DAPI (Sigma-Aldrich, MO, USA) for 30 minutes. Coverslips were mounted on microscope slides with mounting solution containing 90% glycerol, 0.5% N-propyl-gallate and 20 nM Tris, pH=8.0. Primary antibodies were diluted as follows: mouse anti-p21 (Santa Cruz Biotechnology, CA, USA), 1:750; rabbit anti-53BP1 (Cell Signaling Technology, MA, USA), 1:100. Secondary antibodies were diluted as follows: AlexaFluor-488, 1:1500; Alex-Fluor 568, 1:1500 (both from Life Technologies CA, USA).

3.9 Microscope and image analysis

Phase-contrast live cell imaging. Images were acquired on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CoolSnap camera (Photometrics Tucson, USA), XY motorized stage and NanoPiezo Z stage, under controlled atmosphere, temperature and humidity. Neighbour fields (20-50) were imaged every 10 minutes for 2-3 days, using 20x 0.3NA A-Plan objective. Stitching of neighbouring fields was done using the plugin #Stitch Grid" (Stephan Preibisch) from ImageJ/Fiji software.

Automated microscopy. Image fields of 53BP1/p21/cell size mask immunostaining were acquired on IN Cell Analyzer 2000 (GE Healthcare, UK), equipped with a Photometrics CoolSNAP K4 camera and using a Nikon 40x 0.45NA Plan Fluor objective. Fluorescence intensity thresholds were set by eye and used consistently for samples within each experiment.

Image analysis. Fixed cell experiments (senescence-associated biomarkers) were quantified using ImageJ/Fiji software. Mitotic duration, cell cycle duration and cytokinesis failure were quantified from phase-contrast movies.

3.10 Statistical analysis

Sample sizes and statistical tests for each experiment are indicated in the figure legends. All graphs and statistical analysis were performed using GraphPad Prism Software version 6.0. Data were tested for Gaussian distribution using D'Agostino-Pearson omnibus normality test. Student *t* test, Mann-Whitney, two-tailed $\chi 2$ square or one-way ANOVA for multiple comparisons

tests were then applied accordingly. For other comparisons, two-way ANOVA (Sidak's multiple comparisons tests) were used. Statistical significance is represented as follows: ns: $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. Graphs containing error bars shown mean ± SD.

4. RESULTS

Data described on this chapter were presented at:

- 2nd Symposium of Immunomodulation and Cancer and Regeneration (oral communication);
- XLII SPI Annual Meeting (poster).

"Decoding the role of IL-10 in aging"

Ana Catarina Martins, Ana Cardoso, Joana Catarina Macedo, António G. Castro, Isabel Castro, Paulo Vieira, Elsa Logarinho and Margarida Saraiva

June 2017, Porto

4.1 Sustained IL-10 over-expression leads to premature death of pMT-10 mice and to a phenotype marked by hair depigmentation

To fully analyze all the effects imposed by IL-10 in the organism, we started by following a group of pMT-10 mice induced to over-express IL-10. For this, we sustained IL-10 over-expression continuously for 150 days, through the administration of Zn in the drinking water. As controls, three different groups were used – pMT-10 mice non-induced to over-express IL-10 and BL/6 mice fed or not with Zn. This allowed controlling the effect of IL-10 over-expression versus the contribution associated with the pMT-10 genetic background or any side effect of Zn administration. The lifespan analysis of these four groups showed that mice over-expressing IL-10 started to die prematurely (Figure 6A). The medium lifespan was 120 days for the induced pMT-10 group, as compared to all control groups, which did not die during the 150 days of the experimental course. So far we did not determined the precise cause of death of IL-10-expressing mice. During the experimental course, we also monitored the weight of the animals and the overall alterations to their normal appearance and behavior. pMT-10 mice that over-expressed IL-10 maintained their weight over time, instead of gaining weight as the control groups (Figure 6B). Moreover, these animals started to develop patch areas of depigmented/graying hair from day 45 and these alterations became more evident at day 75 (Figure 6C). Both the dorsal and ventral part of the body were affected. Notably, this phenotype was more evident in males, with 9 out of 15 male and 0 out of 6 female mice under experimental conditions displaying it. Control pMT-10 and BL/6 fed or not with Zn showed no hair depigmentation alterations during the time course of the experiment (Figure 6C).

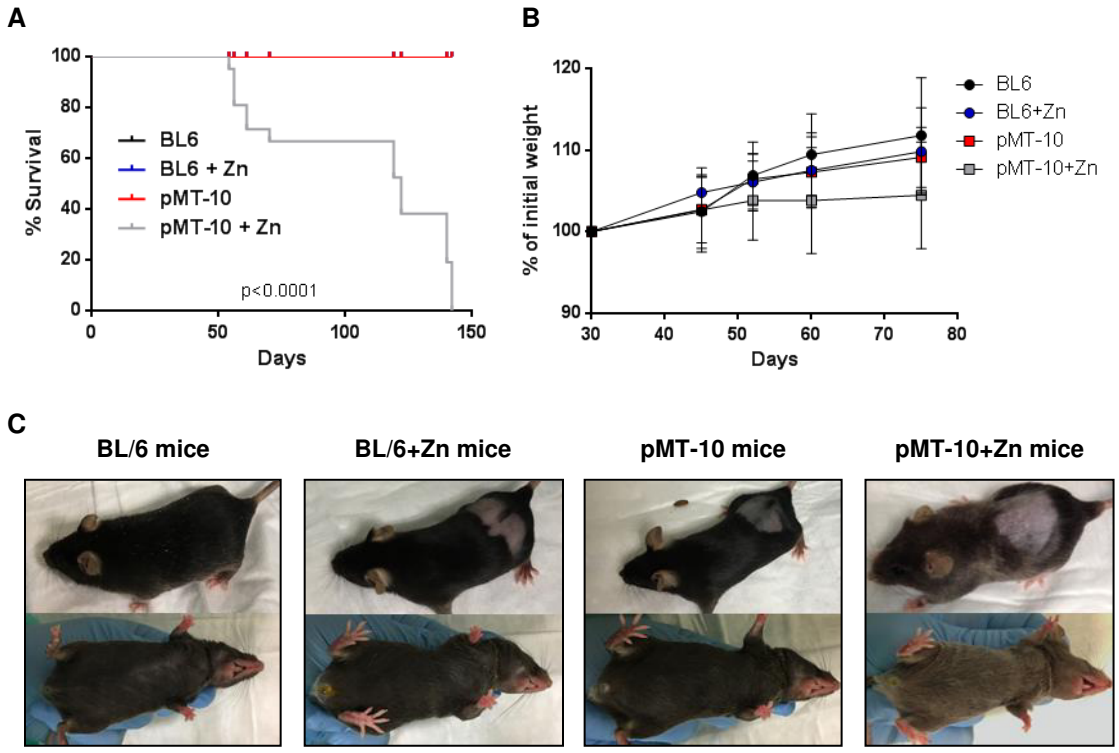

Figure 6. pMT-10 mice over-expressing IL-10 die prematurely and present hair depigmentation. BL/6 and pMT-10 mice were either fed with normal or zinc (Zn)-enriched water for (**A**) 150 days or (**B** and **C**) 75 days. (**A**) Survival analysis. Each curve represents one independent experiment, using 21 independent mice per group. (**B**) Weight curves. Mice were weighted at the indicated time points. Each point represents the mean ± SD for 2 independent experiments, using 5-11 independent mice per group. (**C**) Representative images of mice at day 75 post-Zn administration. Statistical significances were assessed by (**A**) Log-rank (Mantel-Cox) test or (**B**) two-way ANOVA (Sidak's multiple comparisons test). $p<0.001$ indicates significant differences from pMT-10 mice fed with Zn-enriched water.

To control for the production of IL-10 in the different experimental groups, we measured the levels of this cytokine in the serum by immunoassay. Statistically significant higher levels of IL-10 were detected in the serum of pMT-10 mice fed with Zn-enriched water, as compared to all the control groups (Figure 7A). The distribution of the IL-10 levels was widely scattered (1,000 to 20,000 pg/mL). An on-going study from our group has unveiled a role for IL-10 in regulating hematopoiesis, with pMT-10 mice over-expressing IL-10 for 75 days developing an aberrant myeloproliferative phenotype marked by the presence of splenomegaly (Ana Cardoso et al. unpublished). Thus, we looked for spleen alterations in pMT-10 mice over-expressing IL-10 for 75 days. In line with the findings obtained upon short induction of IL-10 (Ana Cardoso et al. unpublished), the histological analysis of the spleen of induced pMT-10 mice revealed a pronounced increase of the spleen weight (Figure 7B) and a structural spleen disorganization

with loss of red and white pulp areas (Figure 7C). All the control groups maintained a normal spleen weight and structural organization (Figure 7B and C).

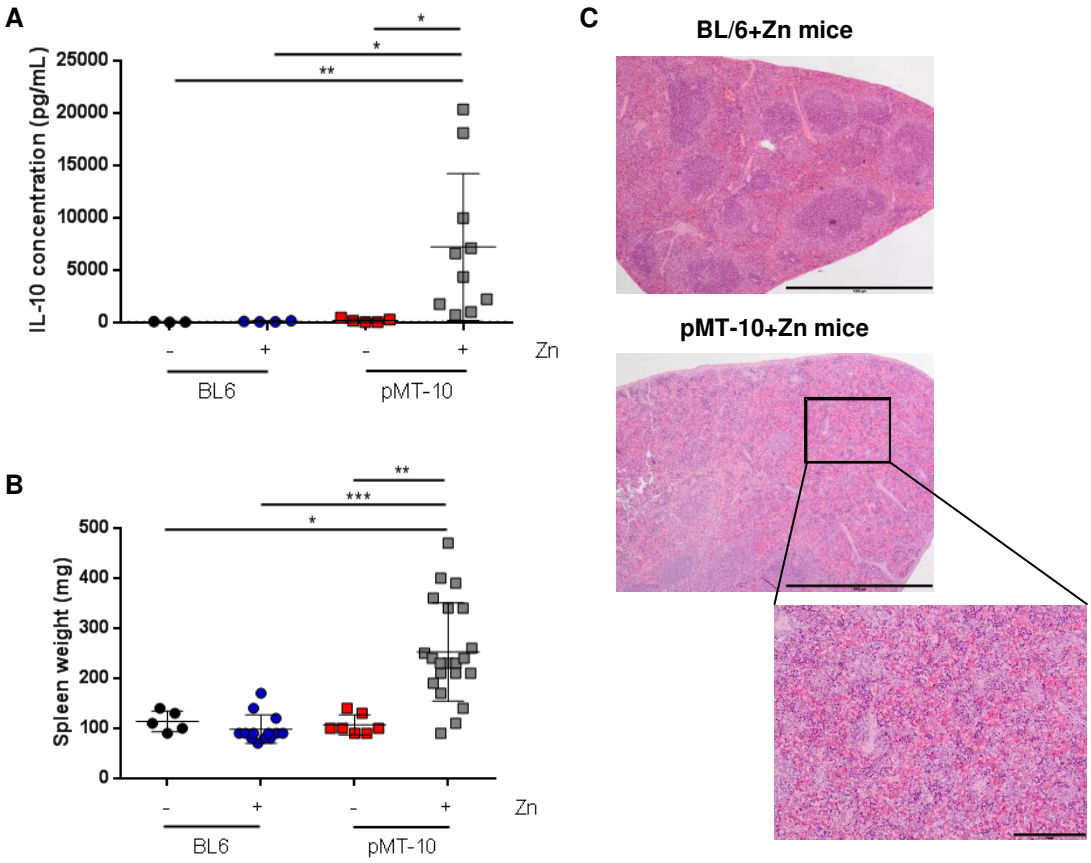

Figure 7. pMT-10 mice over-expressing IL-10 for 75 days present splenomegaly and structural spleen disorganization. BL/6 and pMT-10 mice were either fed for 75 days with normal or zinc (Zn)-enriched water. (**A**) IL-10 expression in the serum. Data is expressed as mean ± SD for one independent experiment, using 5-11 independent mice per group and each dot represents an individual mouse. (**B**) Spleen weight measurement. Data is expressed as mean ± SD for 3 independent experiments, using 3-11 independent mice per group and each dot represents an individual mouse. (**C**) Representative H&E-stained sections of spleen at 40x magnification. Scale bar, 1000µm. Magnified area highlighted from the spleen of pMT-10 mice fed with Zn-enriched water is shown on the right side at 100x magnification. Scale bar, 200µm. Each image is representative of 2 independent experiments, using 5-11 independent mice per group. In both (**A**) and (**B**) statistical differences were assessed by one-way ANOVA (Dunn's multiple comparisons test), *p<0.05; **p<0.01; ***p<0.001 indicates significant differences from pMT-10 mice fed with Zn-enriched water.

As mentioned in the introductory section, the gut is the major IL-10 producing compartment in the induced pMT-10 mouse model (Figure 5C) and IL-10 is an essential cytokine to maintain intestinal homeostasis[120]. Thus, we questioned whether over-expression of IL-10 for 75 days was impacting the gut homeostasis. For this, we performed a histological analysis of the colons and determined the intestinal length for the different experimental groups. By histological examination, no obvious abnormality was found in the gut of BL/6 versus pMT-10 mice fed with Zn (Figure 8A). Consistently with this, no differences were observed in the colon length (Figure

8B). Our results evidenced that, contrary to IL-10 deficiency, exacerbated IL-10 does not impact intestinal homeostasis.

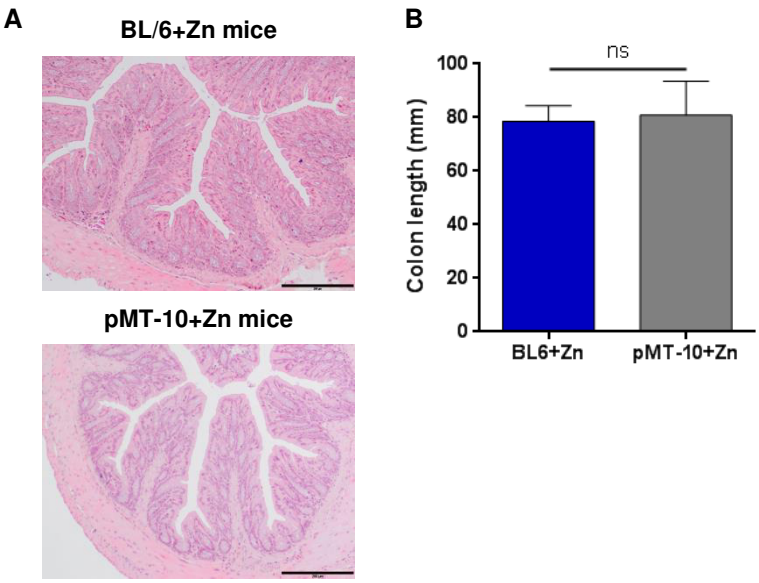

Figure 8. Normal gut histology and colon length in pMT-10 mice over-expressing IL-10. BL/6 and pMT-10 mice were either fed for 75 days with zinc (Zn)-enriched water. (**A**) Representative H&E-stained sections of large bowel at 100x magnification. Scale bar, 200µm. (**B**) Colon length measurement. Each bar represents mean ± SD for 2 independent experiments, using 2-11 independent mice per group. Statistical differences were assessed by student unpaired *t* test; ns, non-significant.

4.2 IL-10 over-expression induces a skin phenotype compatible with an aged skin

Considering the data presented above, it became clear that sustained IL-10 over-expression does impact on the organism homeostasis at different levels. Therefore, we next investigated the extent of the skin phenotype at day 75 of IL-10 over-expression. We focused our study in male mice, as these seemed to be the most affected by IL-10 over-expression.

4.2.1 pMT-10 mice over-expressing IL-10 present structural alterations in the skin

We started by looking for histologic alterations in the skin of pMT-10 mice over-expressing IL-10 for 75 days by performing H&E staining and microscopic observation of the sections. To exclude any effect of Zn, administered to trigger the IL-10 transgene expression, we used wild-

type BL/6 mice fed with Zn as the control group. The study of BL/6 and pMT-10 mice fed with control water is still on-going.

The HF is a mini-organ of the skin that is specialized to grow hair[147]. HF in mammalian skin undergo highly synchronized cyclic phases of growth (anagen), regression (catagen) and quiescence (telogen), ultimately resulting in the generation of a newly formed hair shaft[148,149]. To provide a more in-depth analysis of the effects of IL-10 in the skin, we performed a comprehensive histological characterization of the murine skin at specific stages of the HF cycle: telogen (Figure 9A) and anagen (Figure 9B).

The morphometric analysis of the dorsal skin structure revealed a clear skin thinning of pMT-10 mice over-expressing IL-10 (Figure 9C). Both the dermis and subcutaneous fat layers were significantly thinner in pMT-10 mice over-expressing IL-10 than in control mice (Figure 9D and E, respectively). Histological analysis of the skin also revealed a slight decrease in HF numbers (Figure 9F) in pMT-10 mice over-expressing IL-10, when compared to BL/6 fed with Zn. In addition, skin of the IL-10-exposed pMT-10 mice presented short follicles (Figure 9G).

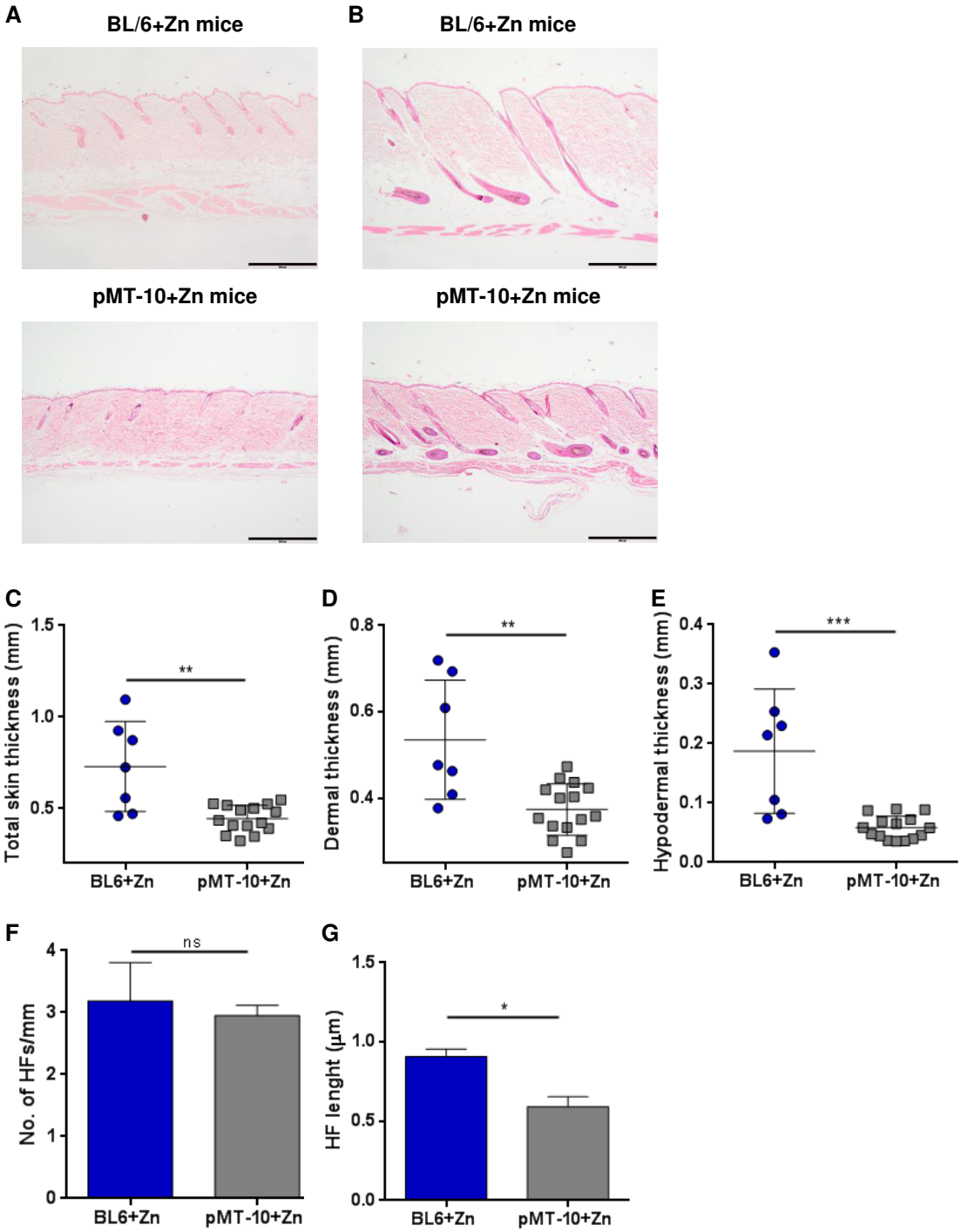

Figure 9. IL-10 over-expression leads to pronounced changes in the dorsal mice skin. BL/6 and pMT-10 mice were fed for 75 days with zinc (Zn)-enriched water. Representative H&E-stained sections of dorsal skin in (**A**) telogen and (**B**) anagen stages at 40x magnification. Scale bar, 500µm. (**C**) Total skin, (**D**) dermal layer and (**E**) hypodermal layer thickness measurement. (**F**) Hair follicle (HF) number per mm. (**G**) HF length measurement. (**C-E**) Data are expressed as mean ± SD for 3 independent experiments, using 3-5 independent mice per group and each dot represents an individual mouse. In both (**F**) and (**G**) each bar represents mean ± SD for 2 independent, using 2-3 mice per group. Statistical differences were assessed by Mann-Whitney test, ns, non-significant; *p<0.05; **p<0.01; ***p<0.001.

As shown above (Figure 6C), the ventral hair of the induced pMT-10 mice was also affected by IL-10 over-expression, showing a general depigmentation pattern. Therefore, we sought to investigate the alterations provoked by IL-10 in the ventral skin of pMT-10 mice. The histologic analysis of skin samples (Figure 10A) showed a reduced skin thickness (Figure 10B) with associated reduction of dermal and hypodermal layers (Fig. 10C and D, respectively), paralleling the observations for dorsal skin. We will now repeat these experiments and increase the sample size, which will allow us to investigate possible HF differences in the ventral skin. Overall, this histological skin phenotype observed, associated with the hair depigmentation, is compatible with an aged skin[147,150,151]. Taken together, these alterations suggest that IL-10 over-expression might underlie a premature skin aging process, something that has never been investigated before.

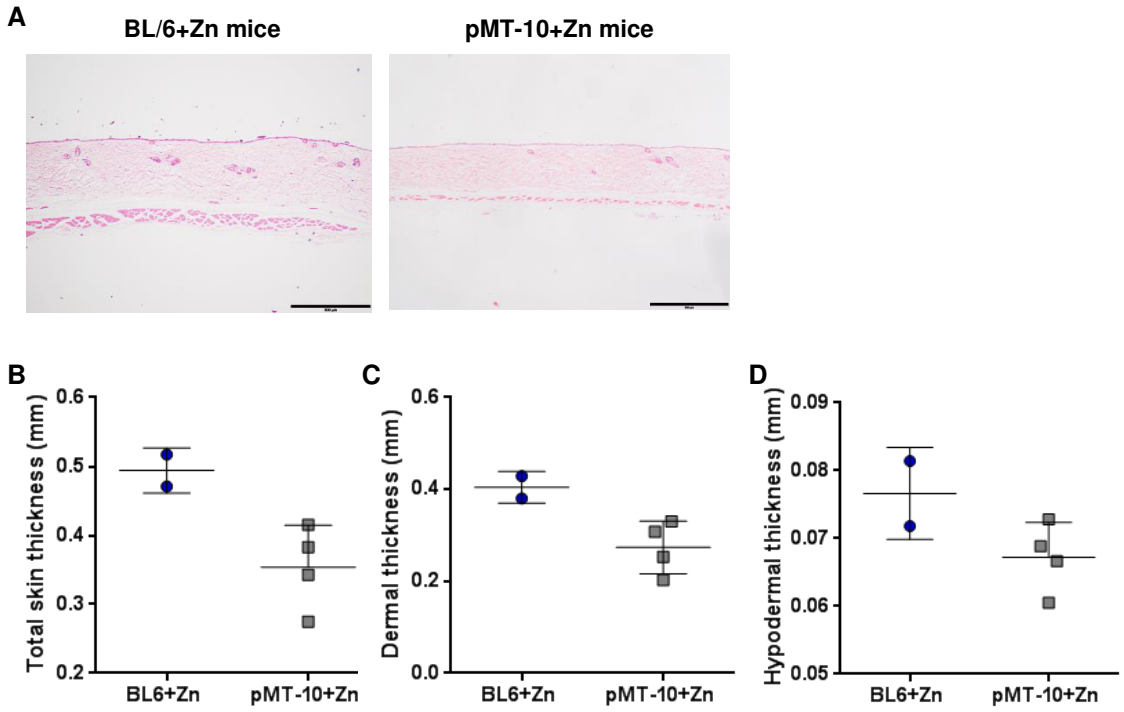

Figure 10. IL-10 over-expression leads to pronounced changes in the ventral mice skin. BL/6 and pMT-10 mice were fed for 75 days with zinc (Zn)-enriched water. (**A**) Representative H&E-stained sections of ventral skin in telogen stage at 40x magnification. Scale bar, 500μm. (**B**) Total skin, (**C**) dermal and (**D**) hypodermal layers thickness measurement. (**B-D**) Data are expressed as mean ± SD for one independent experiment, using 2-4 independent mice per group and each dot represents an individual mouse.

4.2.2 The skin phenotype observed in pMT-10 mice over-expressing IL-10 depends on IL-10R triggering

To assess if these seminal observations are mediated by IL-10, we repeated the experiment using pMT-10 mice crossed with IL-10R$\alpha^{-/-}$ mice. As pMT-10.IL-10R$\alpha^{-/-}$ double transgenic mice are irresponsive to IL-10, any observed effect will be due to unspecific Zn effects in the pMT-10 genetic background. For this set of experiments, we used two experimental groups - pMT-10.IL-10R$\alpha^{-/-}$ fed or not with Zn. Again, we sustained the IL-10 over-expression for 75 days and assessed the development of skin progeroid features and tissue alterations. Gross hair pigmentary abnormalities were not observed in the pMT-10.IL-10R$\alpha^{-/-}$ even upon sustained IL-10 over-expression (Figure 11A). We also evaluated the histological features of the skin and found no evidence of structural alterations in the pMT-10.IL-10R$\alpha^{-/-}$ (Figure 11B). The morphologic quantification of the skin layers revealed no significant differences in the total skin thickness (Figure 11C) as well as in the dermal (Figure 11D) and hypodermal (Figure 11E) layers between pMT-10.IL-10R$\alpha^{-/-}$ mice fed or not with Zn. In all, the double transgenic pMT-10.IL-10R$\alpha^{-/-}$ mice showed a profile very similar with BL/6 mice fed with Zn. Thus, the reported findings associating IL-10 with premature skin aging are mediated by IL-10 activation of the IL-10R.

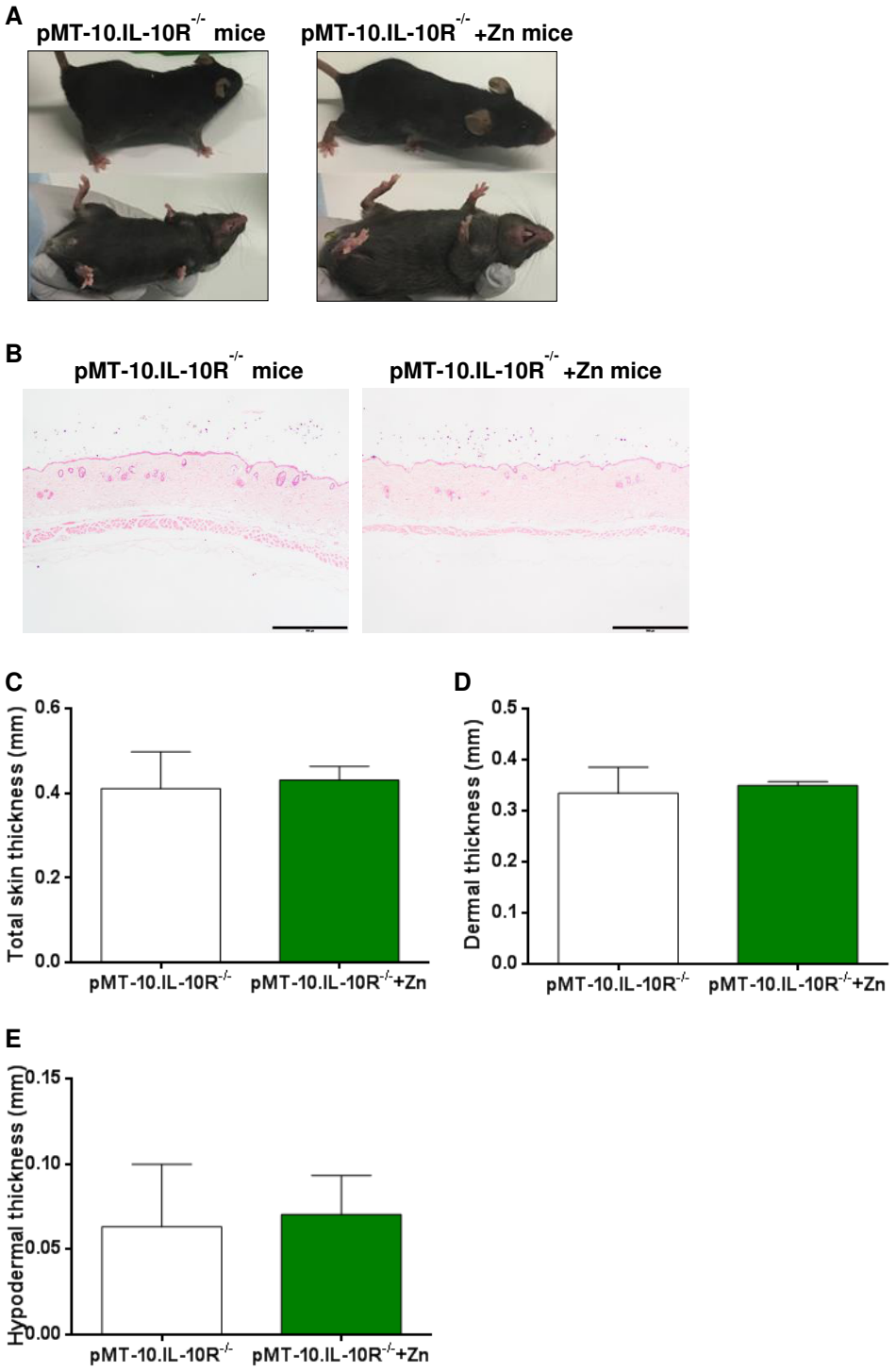

Figure 11. The double transgenic pMT-10.IL-10Rα$^{-/-}$ mice did not develop any obvious alterations in the hair, skin or spleen. pMT-10.IL-10Rα$^{-/-}$ mice were fed for 75 days with normal or zinc (Zn)-enriched water. (**A**) Representative mice images at day 75 post-Zn administration. (**B**) Representative H&E-stained sections of dorsal skin at 40x magnification. Scale bar, 500μm. (**C**) Total skin (**D**) dermal and (**E**) hypodermal layers thickness measurement. (**C-E**) Data are expressed as mean ± SD for one independent experiment, using 2-3 independent mice per group.

4.2.3 IL-10 over-expression affects the ability to repair wounds

Considering the alterations imposed by IL-10 over-expression in the skin, we next investigated the impact of this over-expressing in tissue regeneration, by performing wound healing assays. Skin wound healing is a complex process that requires the interplay of resident epithelial and mesenchymal cells with resident and recruited inflammatory cells, including macrophages, neutrophils and mast cells. This process consists of three stages: inflammation, proliferation and tissue remodeling[152].

For this, we placed two 3-mm punch biopsies in the dorsal skin in pMT-10 mice after Zn administration for 30 and 60 days and monitored the wound size over a period of 8 days. We used BL/6 mice fed with Zn and pMT-10 mice fed with control water as control groups.

Upon 30 days of IL-10 induction, we observed that the wound healing rates in pMT-10 mice induced to over-express IL-10 were substantially delayed by day 2 in comparison to non-exposed ones (Figure 12A). This difference between the two groups tended to slightly decrease at later time points. However, the comparison between pMT-10 and BL/6 fed with Zn indicated that biopsy wounds healed at similar rates, suggesting that the administration of Zn, rather than IL-10 over-expression, might contribute to the observed delay in wounds closure in induced pMT-10 mice.

After 60 days of Zn administration, induced pMT-10 mice exhibited a slight decrease in the percentage of wound closure from day 2 post-wounding, as compared to non-induced animals (Figure 12B). From day 4 post-wound incision BL/6 mice fed with Zn accelerated wound closure, reaching significant difference at day 8 when compared to pMT-10 mice fed with Zn. Collectively, these results raise the question of whether a synergic effect of IL-10 with Zn is in place. In general, induced pMT-10 mice had less ability to repair wounds but this is more evident after a long-term IL-10 induction (60 days) in the late stages of wound closure.

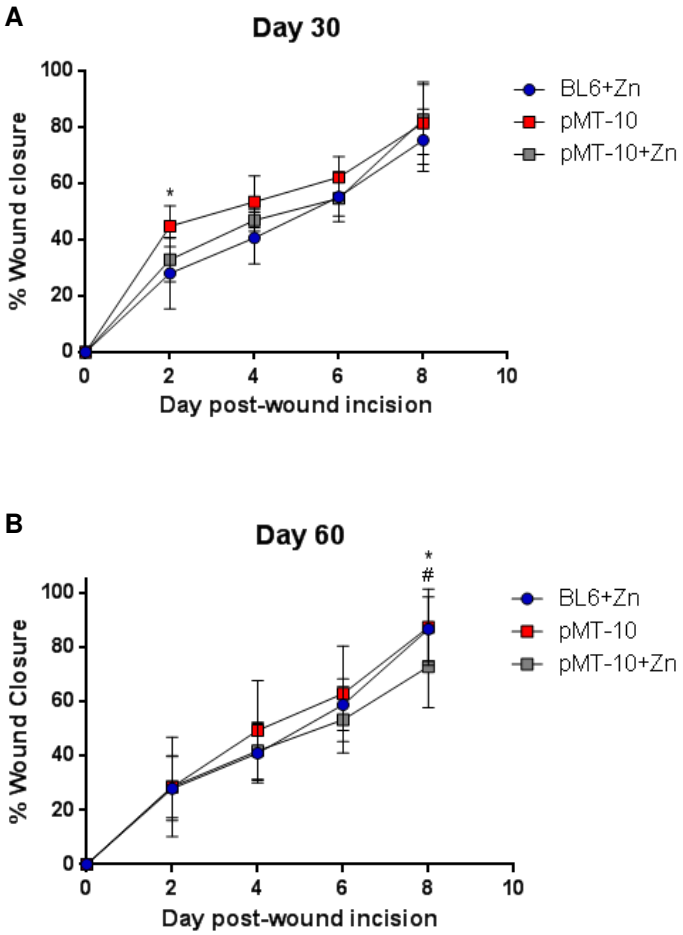

Figure 12. pMT-10 mice over-expressing IL-10 has less ability to repair wounds. BL/6 mice were fed with zinc (Zn)-enriched water and pMT-10 mice were fed with normal or zinc (Zn)-enriched water. Representative kinetics of wound closure at day (**A**) 30 and (**B**) 60 post-Zn administration. Data are expressed as mean ± SD for one or 3 independent experiments, for (**A**) and (**B**), respectively, using 3-5 independent mice per group. Statistical differences were assessed by two-way ANOVA (Sidak's multiple comparisons test). Significant statistical differences from pMT-10 mice fed with Zn-enriched water relative to pMT-10 mice fed with normal water are represented by *; to BL/6 mice fed with Zn-enriched water by #. One symbol, $p<0.05$.

4.2.4 pMT-10 mice over-expressing IL-10 present alterations in hair follicle melanin content

Melanin is the pigment primarily responsible for hair color[153]. Hair pigmentation is accomplished by synthesis of melanin in specialized lysosome-related organelles termed melanosomes and by transfer of these organelles from melanocytes to neighboring keratinocytes[153–156]. Active HF pigmentation is strictly coupled to the anagen phase of the hair cycle, ceases during catagen and is absent throughout telogen[157]. Melanocytes are localized in the epidermis in human skin. In contrast, in adult murine skin, melanocytes generally reside in

the HFs, with the exception of rare epidermal melanocytes found in the tail, footpads and ears. A few dermal melanocytes may also be found, mostly in the ears[158].

The melanin content in the skin is known to change with age[159]. To investigate a relationship between the skin melanin content and the hair depigmentation observed in the induced pMT-10 mice, skin samples were stained with the Masson Fontana method to directly detect melanin. Both groups showed melanin granules typical of growing hair in anagen phase (Figure 13). Microscopic evaluation of BL/6 mice fed with Zn skin demonstrated fully-pigmented HFs, with intense melanization of the hair bulb and hair shaft. However, reduced HF-associated pigment was identified in the IL-10-exposed pMT-10 mice (Figure 13). Thus, these results are in agreement with the hair depigmentation observed in pMT-10 mice that over-expressed IL-10, reinforcing the potential role of IL-10 in premature aging of the skin in mice.

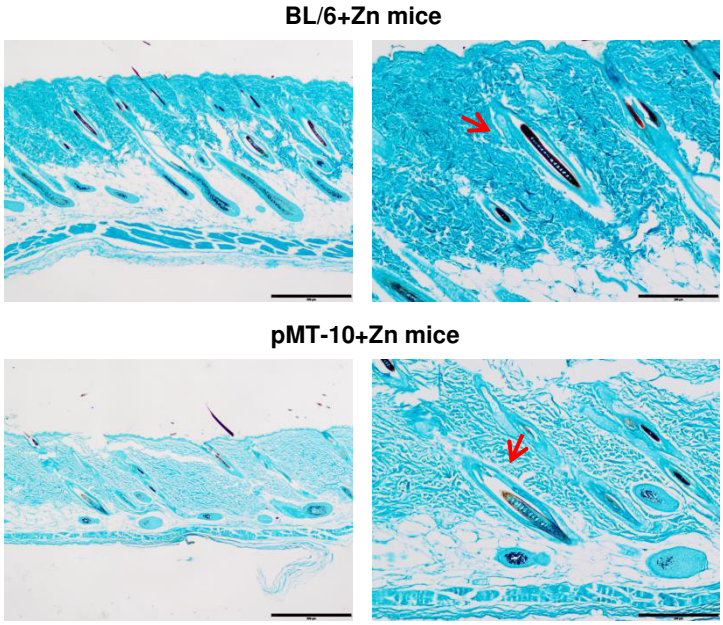

Figure 13. IL-10 over-expression induces alterations in hair follicle melanin content. BL/6 and pMT-10 mice were fed for 75 days with zinc (Zn)-enriched water. Representative Masson Fontana-stained sections of dorsal skin in anagen phase at 40x and 100x magnification for left and right images, respectively. Scale bar, 500µm and 200µm for the left and right images, respectively. Melanin granules in black and pigmented hair follicles (HFs) shown by arrows. Each image is representative of 2 independent experiments, using 2-5 independent mice per group.

4.3 Skin fibroblasts from IL-10-exposed pMT-10 mice divide at a slower rate and show increased frequency of mitotic defects

The faithful transmission of genetic material during cell division is fundamental for cell viability and organismal development[160]. Upon entry in mitosis, replicated interphase chromosomes condense within the nucleus and the previously duplicated centrosomes migrate apart, thereby forming the bipolar spindle (prophase). Nuclear envelope breakdown (NEB) marks the transition to prometaphase[161,162]. During prometaphase, kinetochores (a complex of proteins positioned at the centromere) start to interact with spindle microtubules, such that the chromosomes line up at the equator of the spindle (metaphase). Sister chromatids are then separated and pulled toward the poles, as the kinetochore microtubules shorten (anaphase A) and the poles move apart (anaphase B)[163]. Normally, mitotic duration is defined as the minutes from NEB to anaphase onset, since abscission (complete separation of the two daughter cells from each other) may already occur in interphase G1 phase of the next cell cycle. Once the chromosomes have arrived at the poles, chromatids decondense and nuclear envelopes re-form around the daughter chromosomes (telophase)[162]. Following mitosis, the cell cytoplasm divides in two by cytokinesis[160]. Cell cycle duration correspond to the hours period between the NEB or anaphase onset of the mother cell and the NEB or anaphase onset of the daughter cell respectively. However, in some cases the separation process does not work properly, resulting in unequal distribution of chromosomes[164].

Aneuploidy, defined as an abnormal number of chromosomes, has been linked to aging and the development of age-related diseases[165]. It was recently found that aneuploidy increases with aging due to general dysfunction of the mitotic machinery[166]. Therefore, we hypothesized that cells from pMT-10 mice that over-expressed IL-10 for 75 days might have a general dysfunction of the mitotic apparatus in line with the premature aging phenotype observed in their skin. To probe this hypothesis, we used live cell time-lapse imaging to follow the mitotic pattern of ear fibroblasts collected from BL/6 or pMT-10 mice fed with Zn for 75 days and cultured ex vivo up to 5-6 days. We found a significant increase in the mitotic duration of cells retrieved from IL-10-over-expressing pMT-10 mice, when compared to the control group ones (Figure 14B and C). This mitotic alteration was further accompanied by an increase of the cell cycle duration (Figure 14D). Additionally, cells generated from pMT-10 mice failed cytokinesis at significantly higher

rates than those generated from BL/6 ones (Figure 14A and E). Thus, this ex vivo model evidenced a mitotic and cell cycle delay as a consequence of IL-10 over-expression alongside with a higher rate of mitotic defects, such as cytokinesis failure. Together, these observations suggest that IL-10 triggers loss of mitotic fidelity, in agreement with the role of IL-10 in inducing aging phenotypes.

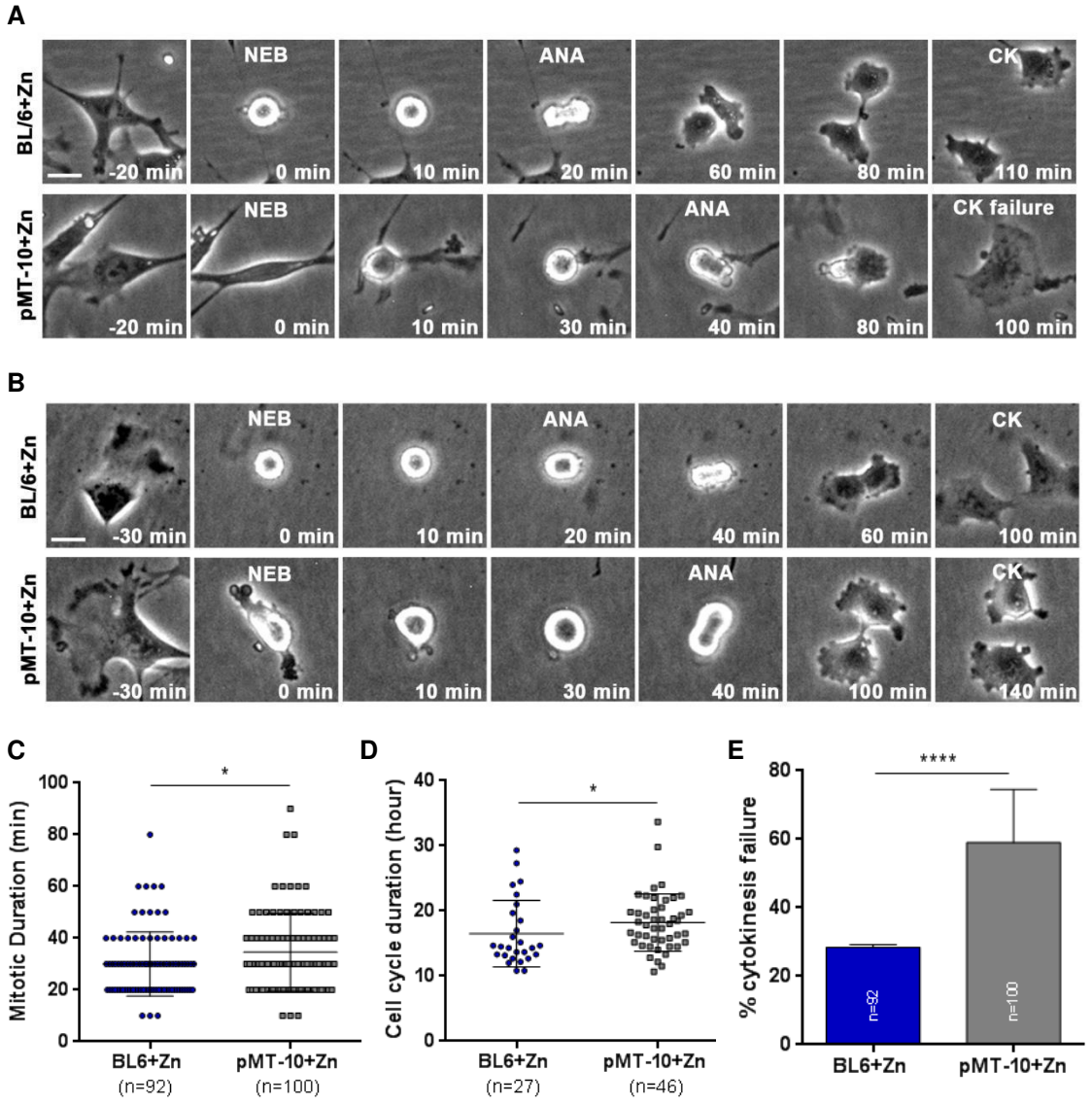

Figure 14. Mitotic defects associated with IL-10 over-expression. Frame series of time-lapse phase-contrast movies of cells collected from BL/6 and pMT-10 mice fed with Zn-enriched water for 75 days, representative of (**A**) cytokinesis failure and (**B**) mitotic duration. Scale bar, 15 µm. (**C**) Mitotic duration of individual fibroblasts. (**D**) Cell cycle duration of individual fibroblasts. (**E**) Rate of cytokinesis failure. (**C-E**) Data are expressed as mean ± SD from one independent experiment, using 2-3 animals per group. Sample size (n) representing the number of individual cells is indicated in each graph and each dot represents an individual cell. Statistical differences were assessed by (**C** and **D**) two tailed $\chi 2$ test or (**E**) Mann-Whitney test; $^{*}p<0.05$, $^{****}p<0.0001$.

The data presented so far place IL-10 as a potential promoter of premature aging. This novel finding is exciting as the cross-talk between the immune system and physiological aging is currently being unveiled, with a growing body of evidence supporting the importance of this cross-talk. Therefore, we decided to further pursue our study by looking for other features of premature aging in pMT-10 mice over-expressing IL-10 and by setting up in vitro systems to investigate the molecular mechanisms underlying the effect of IL-10. Findings from these two research lines are presented in the next sections.

4.4 pMT-10 mice exposed to IL-10 do not develop cataracts or other progeroid hallmarks

We examined the possibility that shortened lifespan of IL-10-induced pMT-10 mice could be accompanied by signs of early aging. For this, we followed the development of overall progeroid traits upon long-term IL-10 over-expression, including lordokyphosis, the presence of bilateral cataracts and kidney alterations[167–170]. None of the BL/6 or pMT-10 mice fed with Zn developed lordokyphosis during the time-line (75 days) (data not shown). Additionally, pMT-10 mice lenses were normal at day 75 post-Zn administration, with no evidence of cataracts (Figure 15A). No cataracts were also observed in wild-type BL/6 mice fed with Zn (Figure 15A). Furthermore, renal sclerosis with glomerulosclerosis, tubular fibrosis and interstitial fibrosis, typical hallmarks of kidney aging[168], were not evidenced in kidney histological sections of induced pMT-10 mice (Figure 15B) indicating preservation of renal function. This, combined with the aged skin phenotype, led us to hypothesize that IL-10 over-expression might play a role in skin aging, instead of in overall organismal aging.

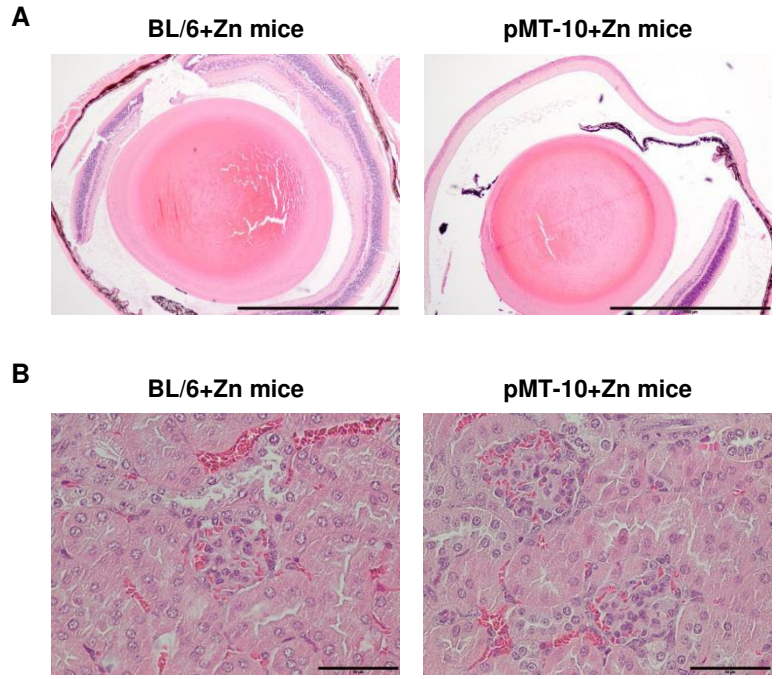

Figure 15. IL-10 over-expression does not led to cataracts or kidney histological alterations. BL/6 and pMT-10 mice were fed for 75 days with zinc (Zn)-enriched water. Representative H&E-stained sections of (**A**) lenses and (**B**) kidney at 40x and 400x magnification, respectively. Scale bars, 1000μm and 50μm respectively. Each image is representative of one independent experiment, using 5-11 independent mice per group.

4.5 Recombinant IL-10 prompted cellular senescence in mouse adult fibroblasts

As noted above, there is mounting evidence that cellular senescence drives aging phenotypes and age-related pathology[40]. Our findings implicating IL-10 in skin aging, led us to question whether IL-10 induces cellular senescence in mouse fibroblasts. To address this, we resorted to an in vitro system, where MAFs were exposed to 50 ng/mL of rIL-10 for different periods of time. Two major cellular processes involved in cellular senescence were investigated by immunofluorescence in MAFs cultured in the presence or absence of rIL-10 for 24 hours, 3, 7 days: i) cell cycle arrest, by testing the activation status of Cdkn1a/p21[171,172]; and ii) DNA damage, by monitoring 53BP1[173].

We started by looking for evidence of cell senescence in cultured fibroblasts over time. We found no evidence of cellular senescence both at 24 hours and 3 days of culture (data not shown). However, upon 7 days of culture, a significant higher percentage of cells containing 53BP1 foci were observed in MAFs stimulated with rIL-10 (47.17%) when compared to their non-stimulated counterparts (38.36%) (Figure 16A). Likewise, rIL-10-stimulated cultures carried

significantly more 53BP1 foci per nucleus than control cells (Figure 16B). Both cultures had mostly one 53BP1 dot per nucleus, but MAFs cultured in the presence of rIL-10 showed a higher frequency of cells with 3, 5 or more 53BP1 foci than untreated cultures. These data suggest a higher incidence of double strand breaks (DSBs) in fibroblasts cultured in a rIL-10-rich medium, thus implicating IL-10 signaling with DSBs and senescence arrest.

We next assessed the expression of p21 by immunofluorescence and found that 19.20% of rIL-10-treated cells versus 11.91% of control cells stained positive for p21 (Figure 16C). These results suggest the involvement of the p53-p21 pathway in the senescence-like growth arrest upon stimulation of rIL-10 for 7 days.

Finally, we combined the 53BP1 and p21 biomarkers to allow for a sensitive and precise quantitative analysis of senescent cells. Cultures exposed to rIL-10 for 7 days were significantly enriched for the frequency of positive cells for both senescence markers (11.68%), as compared with non-exposed cells (5.05%) (Figure 16D). To further understand whether the differences seen were likely associated with IL-10-driven cellular senescence, we evaluated another senescence-related morphologic feature, that is the cell size. Our results evidenced that fibroblasts cultured with rIL-10 underwent drastic changes in morphology, notably an increase in the cell size (Figure 16E).

In all, data from this section implicate IL-10 in MAFs cellular senescence. Moreover, this in vitro system, now set up in our lab, opens new avenues to clarify the molecular mechanisms operating downstream the IL-10R and promoting cellular senescence.

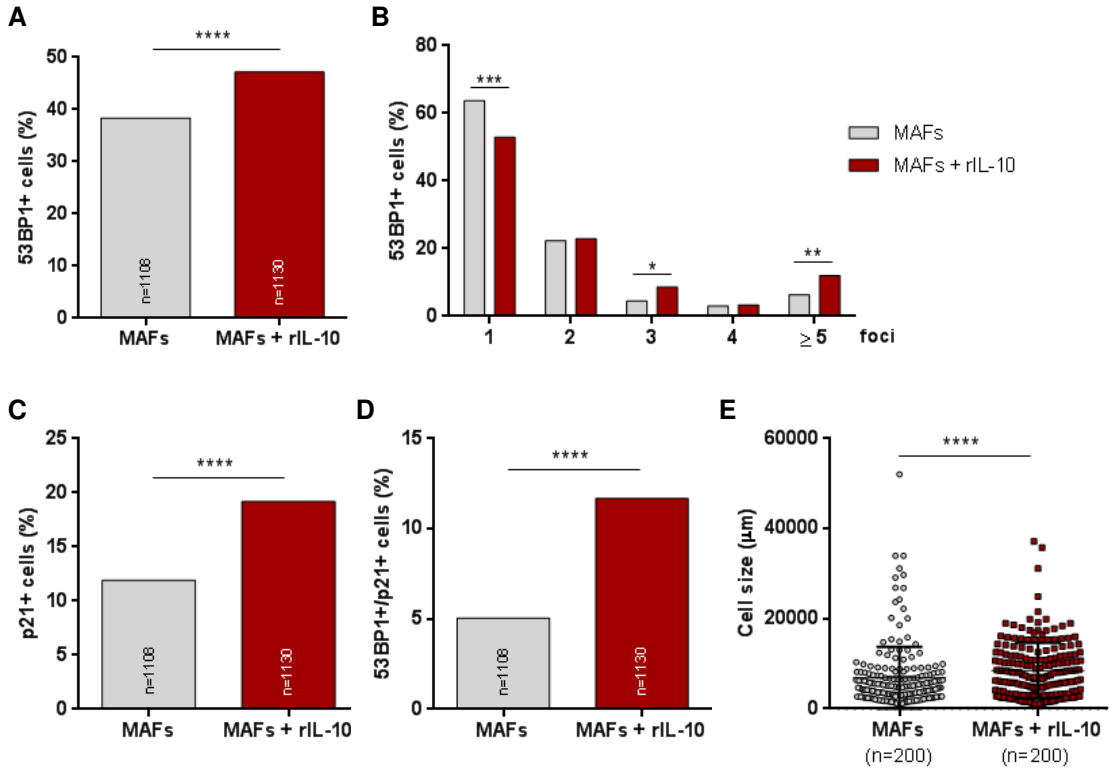

Figure 16. rIL-10 induces cellular senescence in MAFs. (**A**) Percentage of cells staining positive for the senescence marker 53BP1. (**B**) Percentage of cells with 1, 2, 3, 4 and 5 or more 53BP1 foci per nucleus. (**C**) Percentage of cells staining positive for the senescence marker p21 (**D**) Percentage of cells staining positive for both 53BP1 and p21. (**E**) Measurement of cell size. Data is expressed as mean ± SD for one independent experiment. Sample size (n) representing the number of individual cells is indicated in each graph and each dot represents an individual cell. Statistical differences were assessed by (**A-D**) two tailed $\chi 2$ test or (**E**) Mann-Whitney test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5. DISCUSSION

IL-10 is a potent anti-inflammatory cytokine that limits tissue damage, often collateral to the immune response[107]. IL-10 has a great therapeutic potential for inflammatory and autoimmune diseases, and has been tested in clinical trials before[124]. Hallmarks of this are the findings that certain variants of inflammatory bowel disease with onset in childhood are caused by IL-10- and IL-10R-deficiencies[120].

Our study attempts to explore the impact of sustained IL-10 exposure on the organism homeostasis, by resorting to a novel mouse model of inducible IL-10 over-expression (pMT-10)[140]. The pMT-10 mouse model presents itself as a great opportunity to learn more about the impact of high levels of IL-10 on the organism, in a timely-dependent manner and when no other immune alterations are present. This knowledge is important to enhance our understanding of the biology of anti-inflammation and to uncover potential harmful effects of the administration of IL-10 as a therapeutic tool.

As mentioned earlier, previous studies from our laboratory show that in vivo IL-10 over-expression for a short period (15 days) led to an aberrant myelopoiesis (Ana Cardoso et al. unpublished). Specifically, pMT-10 mice over-expressing IL-10 presented increased frequencies of circulating granulocytes and granulocyte-macrophage precursors in the BM and spleen. This augmented myelopoiesis depended on the direct action of IL-10 on hematopoietic progenitors. These changes induced by IL-10 in hematopoiesis resemble those observed in an aged hematopoietic sytem[56].

In this thesis, we sustained the IL-10 over-expression for 150 days and found that pMT-10 mice died prematurely. One of the reported differences between male and female in many species is the death rate, which in males is higher than in females[174]. This is consistent with our data showing that IL-10-exposed pMT-10 female mice are longer-lived than males (data not shown). We also observed that IL-10 over-expression prevents weight increase over-time. Since histological analysis of intestinal sections revealed no abnormalities consistent with a diminished ability to absorb nutrients, it is possible that pMT-10 mice over-expressing IL-10 present a reduced food intake. This is something that we will test in the future. A question that remains open so far relates to the cause of premature death of pMT-10 mice over-expressing IL-10. This is a question to be addressed in the future and our speculation is that it will be mainly related with the abnormal hematopoiesis occurring in these animals.

Most strikingly, the animals induced to over-express IL-10 started to develop a phenotype characterized by hair depigmentation, which is a typical aging phenotype[147]. The hair depigmentation observed in our mice is dependent on gender and on the duration of Zn administration, i.e, on the duration of IL-10 exposure. In contrast to male's hair, female's hair did not appear to be affected by IL-10 over-expression for 75 days. These gender differences affecting the hair of IL-10-exposed pMT-10 mice are in line with the variation found in the mortality patterns, showing that they might be somehow correlated. This gender association is in agreement with other studies, where gender differences were reported in other mouse models of hair depigmentation[175]. Importantly, gender nonconformity has been described in a number of genetic studies of IL-10/longevity association[94]. Thus, in view of our findings, it will be interesting to investigate a possible cross-talk between IL-10, premature aging and hormonal factors[174]. Furthermore, the hair depigmentation phenotype is not observed in 100% of the male group of mice. One possibility for this variation is related with variability in the amount of IL-10 measured in the serum of the animals on day 75 post-induction (Figure 7A). However, we found no direct correlation between the phenotype of hair depigmentation and the amounts of IL-10 in the serum. The variability of IL-10 production is striking and in a way surprising, as we would expect that after 75 days of Zn administration, the levels of IL-10 would be high and constant. However, data from our lab show that the pMT-10 model is tightly controlled by Zn, and therefore the variations observed for IL-10 might reflect the amount of Zn-enriched water consumed by the mice or the time of drinking before the collection[176].

Notably, probed by the hair depigmentation observations, we moved into a deeper characterization of the skin structure upon IL-10 over-expression. We found a thinner dermis and subcutaneous fat cell layer, as well as reduced HF melanin content and visible differences in HF structures, such as diminished HF length and frequency, associated with persistent and sustained IL-10 signaling. It is important to mention that this phenotype was not observed in double transgenic pMT-10.IL-10R$\alpha^{-/-}$ mice, nor in BL/6 mice upon Zn administration. Thus, the reported findings are fully orchestrated by IL-10.

pMT-10 mice fed with Zn-enriched water showed a slightly less ability to repair wounds, but an effect of Zn administration in the BL/6 mice was also evident. In fact, high-dose Zn administration has previously been shown to delay wound healing due to a decrease in cooper absorption, which could result in decreased oxygen delivery to the wound[177–180]. Moreover,

considering the major properties of IL-10 in suppressing the inflammatory response and because IL-10 can be produced by resident skin cells, such as keratinocytes, as well as inflammatory cells involved in the wound healing process[181], it is predictable that IL-10 is involved in the process as a regulator of the inflammatory response. A role for IL-10 in wound healing has already been suggested in earlier studies, showing accelerated wound healing in IL-10 deficient mice[182,183]. This is in line with our findings, as so it is most likely that IL-10 can impact wound repair through an anti-inflammatory response, rather than through its actions in cellular senescence. We will repeat this experiment, creating dorsal wounds of 6 mm, instead of 3 mm, to better understand the impact of IL-10 in the wound healing phases.

In all, the observed histological phenotype is compatible with an aged skin[147,150,151,184]. A major challenge arising from this study is to now clarify the cellular targets underlying this effect of IL-10. An effect in the hair follicle stem cells (HFSCs), which reside in the bulge and sub-bulge area of the HF and give rise to terminally differentiated cells that form the HF[185], is an attractive hypothesis. SC exhaustion is a hallmark of aging[1] involved in the aging of the HF, and consequent hair loss[147]. Furthermore, our study on the role of IL-10 in the hematopoietic process suggests that an effect of IL-10 in precursor cells is in place (Ana Cardoso et al. unpublished). We will now investigate the direct impact of IL-10 on skin SCs, particularly those of the HF, in terms of numbers, location and cell state (occurrence of DNA damage, accumulation of ROS and evaluation of senescence markers).

Although we are suggesting HFSCs as targets, we provide evidence of IL-10 effects on fibroblasts in two different systems. Supported by direct live imaging on fibroblasts of IL-10-exposed pMT-10 mouse, we found a cellular phenotype associated with aging. A recent study has shown evidence for mitotic decline in elderly dividing cells[166]. Interestingly, our data indicate that alongside mitotic delay, IL-10 triggers abnormalities in cytokinesis stage, thus promoting a phenotype compatible with old-aged fibroblasts[166]. As the age-associated mitotic decline is driven by a transcriptional shutdown of mitotic genes in pre-senescence dividing cells exhibiting SASP, it will now be of major importance to assess these features in our model[166]. This will be achieved by resorting to RNA sequencing approaches.

Through the setup of an in vitro experimental model, we also demonstrated that rIL-10 induces premature senescence in mouse fibroblasts through the expression of Cdkn1a/p21. Because rIL-10 appears to generate DNA damage, a persistent DNA damage response could be

the signal to trigger senescence through the p53/Cdkn1a pathway. There is evidence that DNA damage activates IL-10 production in murine keratinocytes[186]. Furthermore, very recent reports included IL-10 and the pro-inflammatory cytokine TNFα and chemokine (C-C) motif 27 (CCL27) in the SASP associated with mitochondrial dysfunction associated-senescence response (MiDAS)[47,187] (Figure 17). MiDAS results from an NADH-AMPK-p53-dependent pathway and elicits growth arrest with a modified SASP[187]. Progeroid mice that rapidly accrue mitochondrial DNA mutations accumulated senescent cells with a MiDAS SASP in vivo, which influenced the differentiation of pre-adipocytes and keratinocytes in cell culture.

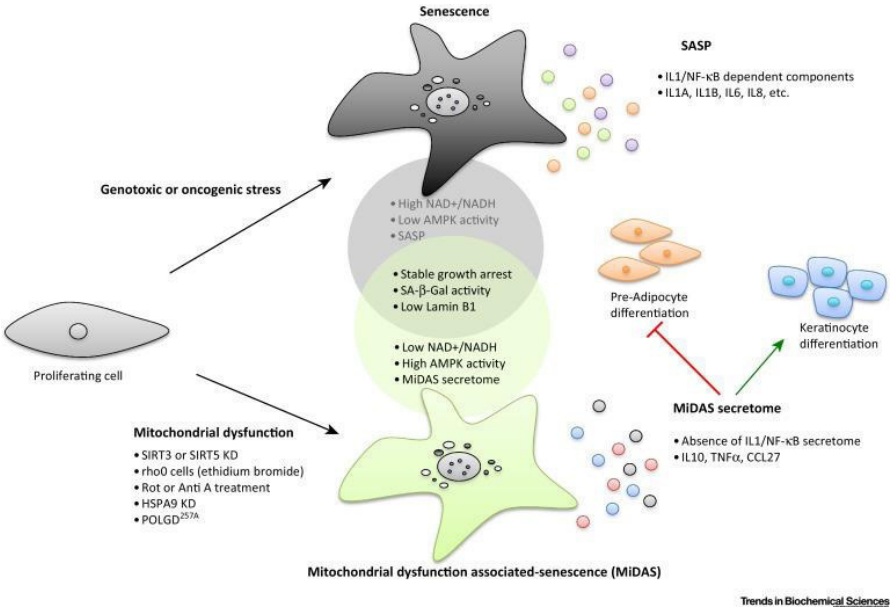

Figure 17. IL-10 is a MiDAS secretome component. Mitochondrial dysfunction associated-senescence (MiDAS) is observed in different genetic backgrounds or in response to treatments that eradicate mitochondria or affect their function. Dysfunctional mitochondria induces cell senescence with a distinct pattern from the caused by genotoxic or oncogenic stress. The MiDAS secretome lacks an interleukin (IL)-1/nuclear factor (NF)-kB-dependent arm and present other factors such as IL-10, tumor necrosis factor (TNF)-α, and chemokine (C-C motif) ligand 27 (CCL27). The secretory phenotype has the ability to inhibit pre-adipocyte and promote keratinocyte differentiation. From Christopher D. Wiley et al.,[187]

These studies suggest IL-10 to be a player in the aging process, however our data show for the first time IL-10-driven aging phenotypes. We will use other senescence biomarkers, including SA-βGal activity[188], for increased lysosomal mass, and ki67, for proliferation arrest, to further validate the senescence phenotype. We will also monitor the activation of several cascades activated downstream the IL-10R or known to be involved in cellular senescence to identify the molecular mechanisms behind the IL-10-driven cellular senescence. Additionally, we will also analyse the conditioned medium of fibroblast cultures stimulated with rIL-10 for 7 days

to identify SASP components secreted through the process of IL-10-induced senescence as well as measure the expression of p16, p21, IL-6 and Mmp3, which compose a minimal transcriptional signature of aging[167].

Progeroid phenotypes observed in other models, such as cataracts and lordokyphosis[169], were not observed in our mouse model. Furthermore, examination of kidney failed to reveal any clear differences in the IL-10-induced pMT-10 mice. One possibility is that pMT-10 mice over-expressing IL-10 for 75 days display an early onset of skin phenotypes associated with aging, with longer time-points being needed for the establishment of a full progeroid phenotype[189]. In support of this, distinct progeroid mouse models show different rates of premature aging[189,190]. Another possibility is the restricted effect of IL-10 over-expression to specific tissues[184]. Therefore, a thorough kinetic evaluation of the pMT-10 mouse model is needed to determine the impact of IL-10 on a broad range of tissues, including the liver, heart, lungs and brain. For this, we will now follow the 4 groups of mice: pMT-10 induced to over-express IL-10, pMT-10 non-induced, and BL/6 fed or not with Zn. The animals will be sex and age matched to avoid further confounding variables, and they will all be obtained and housed under the same conditions to minimize effects linked to, for example, microbiome differences. We will monitor the development of progeroid features, and tissue and cellular age-related alterations in pMT-10 mice exposed to IL-10 for different periods of time. In parallel, the presence of age-associated molecular markers will be investigated. At the systemic level, these include the quantification of hemoglobin, liver and kidney enzymes and sexual hormones in the blood of the animals. At the tissue level, in organs presenting alterations we will investigate the occurrence of DNA damage, the accumulation of ROS and SA-βGal activity. We will also extract RNA from the organs and measure the expression of p16, p21, IL-6 and Mmp3 senescence markers.

Notably, all our observations, from the deviation of the hematopoiesis toward myelopoiesis, the parallel between the observed phenotype and myeloproliferative neoplasms (mostly observed in individuals aged over 50 years[141–145]), to the effects of IL-10 in the skin, strongly incriminate IL-10 as a trigger of premature aging and the development of age-related diseases. Interestingly, these two findings might have as common mechanism, the exhaustion of progenitor cell population. Our findings place IL-10 as a novel regulator of cellular senescence, with impact in organismal aging.

6. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

So far, our findings place IL-10 as a novel regulator of the aging process, particularly in the hair and skin tissue. We also find evidence that IL-10 triggers cellular phenotypes associated with aging in fibroblasts both ex vivo and in vitro. Specifically, we found that IL-10 induces a mitotic decline correlated with a typical age-associated phenotype and we develop an in vitro model of cellular senescence driven by chronic inflammation to investigate potential links between IL-10 and aging.

In all, this work revealed novel and unexpected aspects of the biology of IL-10, specifically its involvement in cellular aging, at least at the skin level. This is important to alert for potential adverse effects of IL-10 as a treatment for inflammatory and autoimmune diseases. Although the contribution of inflammation to the aging process is well accepted[191,192], with older individuals presenting a pro-inflammatory state commonly referred to as inflammaging, an association between anti-inflammation and aging remains controversial. Our findings also contributed to the growing evidence that places the immune system as a key player of aging, by moving the field forward and showing that anti-inflammatory molecules may also drive inflammaging. However, more studies shall be done in order to obtain a full picture of the effects of this cytokine in the organism.

This work will provide functional insight on novel molecules or molecular pathways altered in premature aging that could be of future use as potential targets to rewire the immune response into healthy ageing, whilst at the same time offering potential adverse effects of IL-10 as treatment for inflammatory and autoimmune diseases. Finally, understanding the cross-talk between a balanced immune response and skin remodeling will inform the development of novel therapies for tissue regeneration.

7. REFERENCES

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The dynamics of IL-10-afforded protection during DSS-induced colitis

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Author contribution statement

AC, ACM and GC performed the experiments. AGC and PV made the pMT-10 mice. VM sequenced the genome of the pMT-10 mouse. AC, ACM, AGC, IC, AC, PV and MS planned the experiments and analysed data. AC, AGC, PV and MS wrote the paper.

Keywords

IL-10, Macrophages, Inflammation, Colitis, therapy

Abstract

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Inflammatory bowel disease encompasses a group of chronic-inflammatory conditions of the colon and small intestine. These conditions are characterized by exacerbated inflammation of the organ that greatly affects the quality of life of patients. Molecular mechanisms counteracting this hyper-inflammatory status of the gut offer strategies for therapeutic intervention. Among these regulatory molecules is the anti-inflammatory cytokine interleukin (IL)-10, as shown in mice and humans. Indeed, IL-10 signalling, particularly in macrophages, is essential for intestinal homeostasis. We sought to investigate the temporal profile of IL-10 mediated protection during chemically-induced colitis and which were the underlying mechanisms. Using a novel mouse model of inducible IL-10 over-expression (pMT-10), described here, we show that mice pre-conditioned with IL-10 for 8 days before dextran sulfate sodium (DSS) administration developed a milder colitic phenotype. In IL-10 induced colitic mice, Ly6C cells isolated from the lamina propria showed a decreased inflammatory profile. Because our mouse model leads to transcription of the IL-10 transgene in the bone marrow and elevated seric IL-10 concentration, we investigated whether IL-10 could imprint immune cells in a long-lasting way, thus conferring sustained protection to colitis. We show that this was not the case, as IL-10-afforded protection was only observed if IL-10 induction immediately preceded DSS-mediated colitis. Thus, despite the protection afforded by IL-10 in colitis, novel strategies are required, specifically to achieve long-lasting protection.

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The dynamics of IL-10-afforded protection during DSS-induced colitis

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Keywords: IL-10, macrophages, inflammation, colitis, therapy

Abstract

Inflammatory bowel disease encompasses a group of chronic-inflammatory conditions of the colon and small intestine. These conditions are characterized by exacerbated inflammation of the organ that greatly affects the quality of life of patients. Molecular mechanisms counteracting this hyper-inflammatory status of the gut offer strategies for therapeutic intervention. Among these regulatory molecules is the anti-inflammatory cytokine interleukin (IL)-10, as shown in mice and humans. Indeed, IL-10 signalling, particularly in macrophages, is essential for intestinal homeostasis. We sought to investigate the temporal profile of IL-10 mediated protection during chemically-induced colitis and which were the underlying mechanisms. Using a novel mouse model of inducible IL-10 over-expression (pMT-10), described here, we show that mice pre-conditioned with IL-10 for 8 days before dextran sulfate sodium (DSS) administration developed a milder colitic phenotype. In IL-10 induced colitic mice, Ly6C cells isolated from the *lamina propria* showed a decreased inflammatory profile. Because our mouse model leads to transcription of the IL-10 transgene in the bone marrow and elevated seric IL-10 concentration, we investigated whether IL-10 could imprint immune cells in a long-lasting way, thus conferring sustained protection to colitis. We show that this was not the case, as IL-10-afforded protection was only observed if IL-10 induction immediately preceded DSS-mediated colitis. Thus, despite the protection afforded by IL-10 in colitis, novel strategies are required, specifically to achieve long-lasting protection.

Introduction

Inflammatory bowel disease (IBD) comprises a complex group of inflammatory conditions of the gastrointestinal tract (1) affecting an increasing number of patients worldwide (2-4). Both forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC), result from alterations in the immune homeostasis of the intestinal tissue leading to local uncontrolled inflammation (5, 6). The gut is a very particular site in terms of immune repertoire and regulation, as even in homeostatic conditions constant exposure to antigens occurs (7). Thus, the maintenance of intestinal homeostasis, primarily carried out by intestinal macrophages, requires a constant and fine-tuned balance between the state of tolerance and inflammation (8). In the gut environment, macrophages encounter a plethora of stimuli, from dietary antigens to commensal bacteria, yet, due to their unique tissue specific characteristics, remain tolerant (9). In the pre-disease stage, the epithelial or mucosal barriers become compromised allowing bacteria from the luminal side to invade the *lamina propria* of the gut (10). This event triggers an acute inflammatory response due to the activation of immune cells by direct contact with bacterial products (10). The induced inflammation results either in elimination of the foreign bacterial incursion, or in an exacerbated immune response that can result in tissue damage. The damage caused by deregulated inflammation will perpetuate the activation of effector cells and ultimately lead to the clinical onset of IBD (10, 11).

Epidemiological studies have shown that the etiology of IBD is multifactorial, with genetic predisposition, dysfunctional intestinal barrier and imbalances of the microbiome all contributing to this condition (12-15). Genome-wide association studies (GWAS) revealed that the main genetic alterations associated with IBD are found in genes encoding proteins linked to innate or adaptive immunity, such as the nucleotide-binding oligomerization domain-containing protein 2 (NOD2), Janus kinase (JAK) 2 and tumor necrosis factor superfamily 15 (TNFSF15) (16-18). Other alterations are associated with molecules involved in leukocyte trafficking, regulation of barrier function and secretion of defensins (17). Two reports associate loss-of-function mutations in interleukin (IL)-10 or IL-10R subunits with severe IBD (19, 20). These mutations result in severe enterocolitis, with onset before one year of age, and unresponsiveness to immunosuppressive therapies. The only available therapy for these patients is immune reconstitution with hematopoietic stem cells (21-23). Although complete loss-of-function mutations in IL-10 and IL-10R strongly correlate with IBD, they have an extremely low occurrence rate (19, 24). The most frequent mutations affecting the IL-10 genes associated with IBD are in fact single nucleotide polymorphisms (SNPs) associated with low expression of this molecule (25). However, harboring such mutations does not always translate in low serum levels of IL-10 (23) during the disease stage. This is likely due to the significant increase on the number of IL-10-producing myeloid cells in CD patients (26-29), to the extent that elevated serum levels of IL-10 correlate with disease activity in CD (30-32).

The role of IL-10 in intestinal inflammation is also seen in the mouse model, as IL-10 deficient mice develop microbiome-dependent spontaneous enterocolitis (33). Furthermore, mice with macrophage restricted IL-10R deficiency also develop a spontaneous colitic profile (34), stressing the critical role of the monocyte/ macrophage axis in the immunologic events leading to IBD. Interestingly, it has been shown, in a model of infection that IL-10 can exert a direct effect on monocytes/ macrophages subsets, leading to changes in their inflammatory profile and survival (35). Moreover, IL-10 has been shown to confer protection from hyper-inflammatory states by the induction of the JAK1/STAT3 signalling pathway that suppresses expression of pro-inflammatory mediators and activates expression of anti-inflammatory genes (36).

Taking into account the results obtained in murine models of IL-10 perturbation, the genetic correlation established in humans, and the anti-inflammatory properties of IL-10, this cytokine emerged as a very promising candidate for IBD therapy. However, in IBD patients IL-10-based therapy has not resulted in substantial clinical improvements (37). The main caveats in these clinical trials were the subcutaneous route of administration and the concentration of the recombinant molecule that did not ensure that IL-10 levels reached the mucosal sites, pointing out the importance of novel-locally targeted therapeutic strategies. Furthermore, IL-10 administration to IL-10 deficient murine models only protected from colitis if administered before disease establishment (38).

In this study, we report a novel mouse model of IL-10 over-expression (the pMT-10 mouse) and use it to better explore the mechanisms of immune regulation elicited by IL-10 in the context of intestinal inflammation. We show that a short period of IL-10 over-expression prior to the induction of colitis ameliorates the disease outcome, despite the presence of $CD11b^{+}$ $Ly6C^{+}$ cells in the gut, previously associated with the development of detrimental inflammation. As compared to control animals that do not over-express IL-10, Ly6C cells isolated from the gut *lamina propria* of colitic pMT-10 mice showed a decreased inflammatory profile. Thus, we propose that IL-10 over-expression impaired the response of these cells to the stimulus. In addition to the local effect of IL-10 in controlling exacerbated immune responses, our model allows for the study of IL-10 in imprinting *de novo* generated and circulating monocytes. This is because, constant IL-10 expression is found in specific tissues, in pMT-10 mice, culminating in a systemic effect. Therefore, IL-10 is likely to affect other important compartments, such as the bone marrow (BM) and spleen. IL-10-afforded protection was only seen if IL-10 triggering immediately preceded dextran sulphate sodium (DSS)-induced colitis, thus calling for novel strategies that sustain the effect of IL-10 to offer long-lasting protection.

Material and Methods

Ethics statement

In Portugal 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). Mice were euthanized by CO2 inhalation with efforts to minimize suffering.

In France all animal procedures were approved by the Pasteur Institute Safety Committee and conducted according to French and European Community Institutional guidelines.

Animals

The study involved the use of the following 7-14 week-old-female mice: wild-type C57BL/6, pMT-10 - IL-10 inducible mice, and pMT-10 crossed with IL-10Rα deficient mice (39) (pMT-10.IL-10Rα$^{-/-}$). Food was *ad libitum* for all animals.

Generation of pMT-10 mice

pMT10 mice were generated by A. Gil Castro and Paulo Vieira. Mouse IL-10 cDNA was cloned into the p169ZT vector, which carries a sheep metalloprotein (MT) 1a promoter, a β-globin splice site and a SV40 polyadenylation (polyA) signal. The resulting vector – pMT-10 – (see below Figure 1A) was then injected into C57BL/6 eggs and transgenic founders were identified by PCR using MT and IL-10 specific primers. IL-10 over-expression was induced by giving the mice a 2% sucrose solution with 50mM of zinc (Zn) sulfate to the animals.

DSS-induced Colitis

Mice were fed for 8 days with 3% DSS (TdB consultancy) in the drinking water, and were monitored, daily, for weight loss and disease progression. Colitis progression was measured by the Disease Activity Index (DAI), as previously described (Table 1 and (40)).

Assessment of Intestinal Inflammation

Mice were euthanized on day 8 post-DSS administration or earlier if the symptoms of clinical disease (significant weight loss or diarrhea) became apparent. Samples from colons were immediately fixed in 4% paraformaldehyde (PFA). Then, 5μm paraffin-embedded sections were stained with hematoxylin and eosin, and inflammation was assessed in a blinded fashion using a previously described system (Table 2 and (41)). Samples were graded semi-quantitavely from 0-3 for the four following criteria: (i) degree of epithelial hyperplasia and goblet depletion; (ii) leukocyte infiltration in the *lamina propria*; (iii) area of tissue affected; (iv) and the presence of markers of severe inflammation such as crypt abscesses submucosal inflammation and ulcers. For each sample, criteria scores were added to give an overall inflammation score of 0-12.

156

157 **Cytokine Quantification**

158 IL-10 concentration in the serum was quantified using a commercially available ELISA kit (R&D
159 systems).

160

161 **Preparation of Cell Suspensions**

162 *Lamina propria* leukocytes (LPLs) were prepared as previously described (42). Briefly, LPLs were
163 harvested, dissociated and ressuspended in Hank's Balanced Solution (HBSS) supplemented with 1%
164 fetal calf serum (FCS; Gibco). To isolate LPLs, the colon was flushed with phosphate-buffered saline
165 (PBS; Gibco), opened and cut into 1cm pieces. To eliminate epithelial cells these fragments were
166 incubated at 37°C in Ca- and Mg- free PBS containing 10% FCS and 5.0mM EDTA under strong
167 agitation for 30min. For LPL isolation, the remaining fragments were incubated in RPMI medium
168 with Liberase TL (0,5mg/ml; Roche) for 30min at 37°C. To complete the digestion, the suspension
169 was repeatedly passed through a 10ml syringe for 5 min, filtered through a 40um cell strainer (BD
170 Bioscience) and collected by centrifugation. The cell pellet was ressuspended in 44% Percoll (GE
171 Healthcare), laid over 67% Percoll, and centrifuged at 600g for 20min at 20°C. Cells at the interface
172 were collected, washed in HBSS containing 1% FCS and recovered.

173

174 **Antibodies**

175 Antibodies were conjugated to fluorochromes [FITC, PE, PECy7, APC, APCCy7, Pacific Blue and
176 BV711] and were specific for the following mouse antigens: CD3 (145-2C11; Biolegend), CD11b
177 (M1/70; Sony), CD11c (HL3; Biolegend), CD19 (6D5; Sony), CD45.2 (104; Biolegend), Ly6C
178 (Hk1.4; eBioscience) and Ly6G (RB6-8C5; BD Pharmingen).

179

180 **Cell Sorting and Multiplex Real Time-PCR Analysis**

181 LPLs were FACS-sort purified based on the expression of CD45.2, CD11b and Ly6C, using an Aria
182 III sorter (BD). Cells expressing CD3, CD19, CD11c and Ly6G were excluded. Dead cells were
183 eliminated by exclusion with Propidium iodide (PI). $CD45.2^+$ $CD11b^+$ $Ly6C^+$ cells were sorted
184 directly into a mix of 9µl of CellsDirect One-Step qRT-PCR kit (Life Technologies), containing a
185 mixture of diluted primers (0.05x final concentration, see Supplementary table 1 for references).
186 Preamplified cDNA (18 cycles), was obtained according to the manufacturer's instructions and was
187 diluted 1:5 in TE buffer (pH=8; Ambion). The sample mixture was as follows: diluted cDNA (2.9µl),
188 Sample Loading Reagent (0.32µl; Fluidigm) and Taqman Universal PCR Master Mix (3.5µl; Applied
189 Biosystems). The assay mixture was as follow: Assay Loafing Reagent (Fluidigm) and Taqman Mix.
190 A 48x48 Dynamic Array integrated fluidic circuit (IFC; Fluidigm) was primed with control line fluid,
191 and the chip was loaded with assays and samples with and X IFC Controller (Fluidigm). The
192 experiments were run on a BioMark HD (Fluidigm) for 40 cycles. Gene expression was normalized
193 for *Hprt* and assessed by the $2^{\Delta Ct}$ method.

194

195 **Statistical Analysis**

196 Statistical analysis was performed with the student *t* test or two-way analysis of variance (ANOVA)
197 as indicated in the Figure legends. The analysis was performed with Prism Software (GraphPad).
198 Graphs containing errors bars show means ± SD. Statistical significance is represented as follows: **p*
199 < 0.05, ***p* < 0.01, and ****p*< 0.001.

200

Results

Generation of a novel mouse model of IL-10 over-expression

To study the biological impact of IL-10 over-expression in different settings, we engineered a novel mouse model to allow for inducible IL-10 expression, the pMT-10 mouse (43). For this, a construct containing the IL-10 cDNA under the control of the inducible sheep metalloprotein promoter was introduced in the genome of wild-type BL/6 mice (Figure 1A). Whole genome sequencing reveled a single insertion of the transgene in chromosome 10, between positions 78.813.656 and 78.992844bp (Figure 1A). We estimated, by qRT-PCR, the number of copies of the transgene to be 50-100 (data not shown). The metalloprotein promoter is activated in the presence of 50mM of Zn in the organism, administered in the drinking water. Kinetic analysis of IL-10 in the serum of pMT-10 mice fed with Zn-enriched water showed a rapid increase of circulating IL-10 (Figure 1B). Indeed, as soon as day 3 after IL-10 induction, the levels of this cytokine in the serum were very high (7-12ng/ml) (Figure 1B). Moreover, suspending the Zn administration led to a drop in IL-10 in sera to levels below detection in only 4 days (Figure 1B). As expected, circulating IL-10 was undetectable in pMT-10 mice fed with normal water (Figure 1B). Transcriptomic analysis of different organs and cellular compartments of induced pMT-10 mice revealed that the expression of the exogenous IL-10 cDNA was restricted to $CD45^{-}TER119^{-}$ cells from the small intestine (SI), skin and, to a less extent, BM (Figure 1C). IL-10 was not detected in the other organs analised (liver, spleen, kidney, choroid plexus, lung and colon; data not shown). Thus, the pMT-10 mouse model allows for timely controlled IL-10 over-expression in specific anatomic locations, accompanied by a strong increase of the levels of this cytokine in the serum.

DSS-induced colitis is ameliorated in IL-10 pre-exposed mice

Despite the clear link between low levels of IL-10 and susceptibility to colitis in human (44) as well as in mouse models (33, 34), administration of IL-10 to treat this condition showed only limited effects (37). A possible reason may be the poor accessibility of IL-10 to the site of inflammation. In this context, and in view of the high expression seen in the SI of induced mice, the pMT-10 mouse model offers an opportunity to further address the effects of IL-10 expression in the gut in the context of colitis. For this, we used the DSS experimental model, a highly reliable and reproducible way of causing UC-like symptoms in the mouse model by inducing acute inflammation with the recruitment of inflammatory cells (45). In our experimental setting, wild-type BL/6 mice started to show signs of disease from day 3-4 after administration of 3% DSS in the drinking water (S1 Figure). pMT-10 mice showed a disease profile very similar to BL/6 mice (S1 Figure).

We investigated the impact of IL-10 over-expression prior to DSS-induced colitis. For this, pMT-10 mice were induced to over-express IL-10 for 8 days, before initiation of DSS administration (Figure 2A). As compared to control pMT-10, mice pre-conditioned with IL-10 showed significantly lower DAI after day 4 (Figure 2B) indicating that IL-10 conferred partial protection. Although no differences were observed in the colon length of induced versus non-induced mice (Figure 2C), histologic analysis of the organ revealed that IL-10 protected against DSS-induced colitis, with reduced inflammatory infiltrates, reduced architectural distortion (characterized by crypt shortening

and branching) and less ulceration (Figure 2D). Quantification of the alterations by histological score revealed a significant improvement of the pMT-10 mice over-expressing IL-10 as compared to the control group (Figure 2E). Thus, our data showed that IL-10 over-expression prior to intestinal insult was sufficient to afford a degree of protection from DSS-induced colitis.

IL-10 and Zn work in a concerted way to diminish colitis symptoms

As a gastrointestinal disease, it is not surprising that diet plays an important role in the pathogenesis of IBD, directly impacting the maintenance of the intestinal barrier, gut microbiome and immune responses (46-48). Zn in particular, is essential for intestinal homeostasis, due to the existence of several Zn-dependent antioxidant enzymes, such as superoxide dismutase, that neutralize free radical production (49, 50). Several studies show that high dietary intake of Zn is associated with a reduced risk of relapse of CD (51, 52). Therefore, we hypothesized that the Zn administered to trigger IL-10 expression might contribute to the observed protection against DSS-induced colitis. To assess this hypothesis, we applied the same experimental protocol as before to BL/6 wild type or pMT-10 mice (Figure 3A). Again, significant differences were observed as early as day 5 post-DSS administration between the DAI of mice over-expressing IL-10 and those that do not (Figure 3B). However, by day 7 of DSS administration, significant lower DAI was observed in BL/6 mice fed with Zn-enriched water as compared to control (Figure 3B). A statistically significant increase was observed in the colon length of mice pre-exposed to IL-10 and Zn, but not on BL/6 only exposed to Zn in comparison to BL/6 controls (Figure 3C). Histologic analysis of the organ showed an improvement in pMT-10 mice pre-exposed to IL-10 as compared to BL/6, exposed or not to Zn (Figure 3D), although the histological score did not reach statistical significance (Figure 3E). Overall, the maximal protection was observed for pMT-10 mice over-expressing IL-10, which suggests a synergistic effect of IL-10 and Zn in the amelioration of the disease. To investigate this issue, we repeated the experiment using pMT-10 mice crossed with IL-10Rα$^{-/-}$ mice. Since pMT-10.IL-10Rα$^{-/-}$ double mutant mice are unresponsive to IL-10, the effects observed would only be due to Zn administration. In these mice, we observed an accelerated disease progression upon DSS administration, with an elevated DAI score as early as day 3, in line with the known role of IL-10 in controlling the disease (Figure 3F). Furthermore, we found that in these mice Zn administration failed to confer protection against DSS-induced colitis (Figure 3F). In all, our findings support the notion that the protection afforded by Zn requires IL-10 signalling.

Pre-exposure to IL-10 promotes a more controlled inflammatory response

Previous studies have shown that monocytes and macrophages are the major effector subsets of colonic inflammation (34, 53). Mice with macrophage-specific IL-10R deficiency develop a spontaneous colitic profile, emphasizing the importance of IL-10 in regulating the macrophage response to prevent uncontrolled inflammation (34). Thus, we next investigated whether IL-10 ameliorated DSS-induced colitis by restricting the monocyte/macrophage response. Considering that Zn administration also improved the outcome of DSS-induced colitis in BL/6 mice, we compared the transcriptional profile of monocytes/macrophages from BL/6 or pMT-10 mice pre-exposed to Zn and subjected to DSS administration for 4 days. We chose this time point, since signs of colitis induced by DSS in both BL/6 and pMT-10 mice only become obvious after day 4 of DSS administration. Thus, BL/6 and pMT-10 mice were fed with Zn-enriched water for 8 days and then received DSS for 4 days (Figure 4A). At this time point, Ly6C^{+} cells from each mouse from the different groups were

FACS purified (Figure 4B). Expression of 22 genes (S1 Table) associated with the uncontrolled immune response developed in IBD were analysed by multiplex RT-PCR. All samples, from both groups, expressed three house-keeping genes (*Hprt, Actb* and *Gapdh*). Of the 22 genes analysed, we failed to detect expression of 9 (*Il4*, *Il9*, *Il12α*, *Il12β*, *Il13*, *Il17*, *Il23*, *Ifnγ* and *Cx3cl1)* in Ly6C$^+$ cells isolated from the *lamina propria* in both groups. We detected expression of the 10 remaining genes in Ly6C$^+$ cells, in both BL/6 and pMT-10 mice after DSS administration (Figure 4C), but no expression in the absence of insult (data not shown). Thus, Ly6C$^+$ cells alter their expression profile in response to DSS insult. Most interestingly, on day 4 post-DSS administration, Ly6C$^+$ cells isolated from induced pMT-10 mice presented an overall less inflammatory profile than those isolated from BL/6 mice (Figure 4C). In the case of *Tnfα* and *Cd86*, the differences observed between the 2 mouse groups were statistically significant. In all, these findings suggest that exposure to IL-10 before DSS induction acts by preventing an inflammatory profile in Ly6C$^+$ cells. Of note, the frequency of inflammatory macrophages recruited to the inflamed gut was similar between the two groups, and the same was true for CD3 T cells and CD19 B cells, showing that IL-10 over-expression does not impact the recruitment of immune cells to the gut (Figure 4D).

IL-10 protection against DSS induced colitis is not long lasting

In our mouse model, IL-10 is also over-expressed in the BM and is found at high levels in the serum, possibly creating an anti-inflammatory environment that could pre-condition de novo generated or circulating monocytes. In this setting, the circulating monocytes could thus be educated to be less responsive once recruited to the colon during DSS-induced colitis. To study this possibility we combined a period of IL-10 over-expression with a resting period of 21 days prior to DSS administration (Figure 5A). We chose 21 days because previous reports showing that resident intestinal macrophages have a life span of approximately 3 weeks (54). After reversion of IL-10 expression to basal levels we observed no protection against DSS-induced colitis, with no differences between the two groups regarding DAI (Figure 5B), colon length (Figure 5C) or histology (Figure 5E and 5F). Thus, we conclude that IL-10 over-expression, over a period of 8 days, does not confer long-lasting protection against intestinal inflammation.

Discussion

Despite the fact that IBD is a treatable condition, there are many limitations to the therapeutic approaches currently available (5). Major obstacles in this context are the heterogeneity of the disease, which implies that dosage and schedule may differ across disease conditions, and the requirement of a localized action of the therapeutic agent. In view of the strong immune component associated with disease development, it is not surprising that therapeutic manipulations of the immune response have been widely sought approaches to tackle IBD. Indeed, a commonly used therapy for IBD is the administration of anti-tumor necrosis factor alpha (TNF-α) antibodies (55). However, in line with the above mentioned limitations, up to one-third of IBD patients do not respond to this therapy, and those who respond eventually develop some degree of intolerance to the medication (56). In this context, several animal models of IBD, both spontaneous and experimentally induced (such as DSS), were developed to investigate the role of various factors on the pathogenesis of the disease and to evaluate the different therapeutic options. A molecule that has been widely studied in the context of IBD is IL-10. This cytokine keeps intestinal inflammation in check by exerting a direct effect on monocyte/ macrophage populations (34). Thus, it is not surprising that IL-10-based therapies have been tested in IBD. However, both in human (57-60) and mouse models (38), administration of IL-10 did not significantly improve intestinal inflammation, perhaps in part due to the fact that administered IL-10 did not reach the inflamed tissue.

In this study, we report a novel transgenic mouse model of inducible IL-10 over-expression, the pMT-10 mice, in which high IL-10 transcription is observed in the intestine, skin and BM. Upon induction of the transgene, high levels of IL-10 are detected also in the serum. Taking advantage of this novel mouse model, we investigated the dynamics of IL-10 afforded protection during DSS-induced colitis.

We found that induction of IL-10 prior to DSS administration impacted the progression of colitis, not only by keeping the disease score (measured by DAI) lower than that observed in the absence of IL-10, but also by decreasing the histologic damage to the tissue. Because the induction of IL-10 expression in pMT-10 mice relies on the increased dietary intake of Zn, and since this metal has been previously described as protective in the context of intestinal inflammation (61, 62), we investigated the impact of dietary Zn intake in wild-type animals. We show that Zn administration *per se* had some effect in reducing the severity of colitis, but this effect occurred both later and to a lower extent than that observed for the combined condition Zn+IL-10. In addition, the protective effect of Zn failed to overcome the exacerbated colitis observed in mice that did not respond to endogenous IL-10. Of note, a former study using a similar mouse model for TGF-β induction showed no effect of Zn in ameliorating DSS-induced colitis (63). We further show that exposure to IL-10 prior to colitis induction and establishment contributed to a decreased inflammatory response, which was more pronounced that the effect achieved upon Zn intake. This conclusion was derived from transcriptional studies on sort-purified $Ly6C^{+}$ cells, re-affirming both the critical role of these cells on intestinal inflammation (53) and that of IL-10 in regulating their inflammatory responses (34). Although previous studies have shown that Zn deficiency causes functional defects of peripheral blood monocytes and tissue macrophages and increases the concentration of inflammatory cytokines and oxidative stress (64, 65), that did not appear to be the mechanism of Zn-mediated protection in our model. One possibility is that Zn may contribute to diminish the amount of free radical species generated during acute colitis which contribute to protein, DNA chain and lipid damage (66). As IBD patients often have a Zn deficiency and respond well to Zn supplementation therapy (50), the exploitation of combined IL-10 and Zn therapies may be worth considering. In line with this, the benefits of combined Zn and anti-TNF therapy were previously described (61).

The pMT-10 mouse model allows for local, as well as systemic, IL-10 over-expression. The fact that we detected increased transcription of the IL-10 transgene in the BM and elevated levels of seric IL-10 led us to hypothesize that pre-exposure to IL-10 might induce long-lasting transcriptional changes in circulating monocytes, for example through epigenetic imprinting. If this were the case, we might be able to educate these cells to gain long-lasting tolerance to DSS-induced colitis. However, this did not seem to be the case, as the protective effects of IL-10 were not sustained over time, implying that IL-10 presence at the time of insult is necessary to prevent colitis. Therefore, inducing IL-10 expression in our mouse model at the beginning of disease would be of interest. Unfortunately, we were unable to explore this possibility because the Zn necessary to activate the transgene precipitates in the presence of DSS, when both are provided in the drinking water.

In conclusion, we herein present a novel mouse model of inducible IL-10 over-expression. We also show the potential of this model for the study of the IL-10 biology in the specific setting of DSS-induced colitis. Our data further support the protective role for IL-10 in intestinal inflammation, showing that this cytokine delays disease progression even when delivered before DSS administration. However, the effect is not long-lasting, which calls for alternative approaches to prevent IBD.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors and Contributions

AC, ACM and GC performed the experiments. AGC and PV made the pMT-10 mice. VM sequenced the genome of the pMT-10 mouse. AC, ACM, AGC, IC, AC, PV and MS planned the experiments and analysed data. AC, AGC, PV and MS wrote the paper.

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583

584

585 **Figure Legends**

586

587 **Figure1. A novel mouse model for inducible IL-10 expression: pMT-10 mice.** (**A**) Schematic
588 representation showing the targeting vector and insertion site. (**B**) Kinetics of IL-10 over-expression
589 in the serum at different time points post Zn administration and Zn withdrawal. pMT-10 mice were
590 fed with normal (pMT-10-Zn) or Zn-enriched (pMT-10+Zn) water and at the indicated time points
591 blood was harvested and the amount of IL-10 in serum measured by immunoassay. (**C**) qRT-PCR
592 identified $CD45^-$ $TER119^-$ cell subsets from skin, BM and small intestine (SI) as the main producers
593 of IL-10 in pMT-10 mice fed for 8 days with Zn-enriched water. In both (**B**) and (**C**), each point or
594 bar represents the Mean±SD for 3 independent mice. Data were analysed with [B] two-way ANOVA
595 (Sidak's multiple comparisons test) or (**C**) student *t* test, **p*<0.05; ***p*<0.01; ****p*<0.001.

596

597 **Figure 2. DSS induced pathology is ameliorated by pre-exposure to IL-10.** (**A**) pMT-10 mice
598 were fed for 8 days with normal (pMT-10-Zn) or Zn-enriched (pMT-10+Zn) water, followed by 8
599 days of 3% DSS administration also in the drinking water. (**B**) Disease progression based on DAI
600 parameters was registered every day for 8 days. (**C**) Colon length measurement at day 8 of DSS
601 administration. (**D**) Representative H&E-stained sections of large bowel at 40x magnification (Scale
602 bar = 200µm). (**E**) Colitis scores derived from evaluation of colon and cecum from either group.
603 Each point or bar represents the Mean±SD for 3-5 independent mice, in 2 independent experiments.
604 Data were analysed with (**B**) two-way ANOVA (Sidak's multiple comparisons test) or (**C**) and (**E**)
605 student *t* test, ***p*<0.01; ****p*<0.001.

606

607 **Figure 3. Protection from DSS-induced colitis in induced pMT-10 mice depends on IL-10**
608 **receptor signalling, although Zn on its own also displays a partly protective effect**. (**A**) BL/6 and
609 pMT-10.IL-10R$\alpha^{-/-}$ mice were fed with normal water (BL/6-Zn and pMT-10.IL-10R$\alpha^{-/-}$-Zn
610 respectively) or, were fed for 8 days with Zn-enriched water (BL/6+Zn, pMT-10+Zn pMT-10.IL-
611 10R$\alpha^{-/-}$+Zn), followed by 8 days of 3% DSS administration also in the drinking water. (**B**) and (**F**)
612 Disease progression based on DAI parameters was registered every day for 8 days. (**C**) Colon length
613 measurement at day 8 of DSS administration. (**D**) Representative H&E-stained sections of large
614 bowel at 40x magnification (Scale bar = 200µm). (**E**) Colitis scores derived from evaluation of colon
615 and cecum from either group. Each point or bar represents the Mean±SD for 3-7 independent mice,
616 in 2 independent experiments. Data were analysed with (**B**) and (**F**) two-way ANOVA (Sidak's
617 multiple comparisons test) or (**C**) and (**E**) student *t* test, **p*<0.05; ***p*<0.01; ****p*<0.001, $^{\#\#\#}$p<0.001.
618 * compare BL/6-Zn against pMT-10+Zn; #, compare BL/6+Zn against pMT-10+Zn.

619

620 **Figure 4. $Ly6C^+$ cells pre-exposed to IL-10 reveal a less inflammatory profile upon DSS-**
621 **induced colitis than those pre-exposed to Zn.** (**A**) pMT-10 or BL/6 mice were fed with Zn-enriched
622 water for 8 days, followed by 4 days of 3% DSS administration. (**B**) At the end of the DSS treatment,
623 LPLs were isolated and $Ly6C^+$ cells sort-purified. Shown is the gating strategy for $Ly6C^+$ cells
624 purification. (**C**) Sort-purified $Ly6C^+$ cells (n=25 cells) were analysed by qRT-PCR for a total of 22
625 genes using the BioMark HD system. Samples were normalized for *Hprt* expression. Represented is
626 the expression heatmap compiling the genes which expression was detected in either mouse group.

Each heatmap rectangle represents the mean of gene expression obtained for cells isolated from 5 independent mice. (**D**) The frequency of the different leukocyte subsets was determined upon staining of LPLs for $Ly6C^{+}$ cell sorting. Each bar represents the Mean±SD for 5 independent mice. Data were analysed with student *t* test, **p*<0.05.

Figure 5. The IL-10 protection conferred against DSS induced colitis is not long lasting. (**A**) pMT-10 mice were fed with control (pMT-10-Zn) or Zn-enriched (pMT-10+Zn) water for 8 days, followed by a 21-day resting period where only normal water was available, and by 8 days of 3% DSS. (**B**) Disease progression based on DAI parameters was registered every day for 8 days. (**C**) Colon length measurement at day 8 of DSS administration. (**D**) Representative H&E-stained sections of large bowel at 40x magnification (Scale bar = 200μm). (**E**) Colitis scores derived from evaluation of colon and cecum from both groups. Each point or bar represents the Mean±SD for 3-6 independent mice, in 2 independent experiments. Data were analysed with (**B**) two-way ANOVA (Sidak's multiple comparisons test) or (**C**) and (**E**) student *t* test.

641 **Tables**

642

643 **Table 1. DAI parameters.** DAI is obtained by the sum of each individual score.

Score	Weight Loss	Stool Consistency	Bleeding
0	No loss	Normal	No blood
1	1-5%	Mild-Soft	Brown Colour
2	5-10%	Very soft	Reddish Colour
3	10-20%	Diarrhea	Bloody Stool
4	>20%		Gross Bleeding

644

645 **Table 2. Parameters for histological analysis of colitis severity**. The final score is obtained by the
646 sum of individual scores. Markers of severe inflammation included ulceration and crypt abscesses.

Score	Epithelial Hyperplasia & Goblet Depletion	Leukocyte infiltration in the *Lamina Propria*	Area Affected	Markers of Severe Inflammation
0	None	None/Rare	None	None
1	Minimal	Increase	1/3	Minimal
2	Mild	Confluent	2/3	Increased
3	Marked	Transmural	All	Confluent

647

Figure 1.TIF

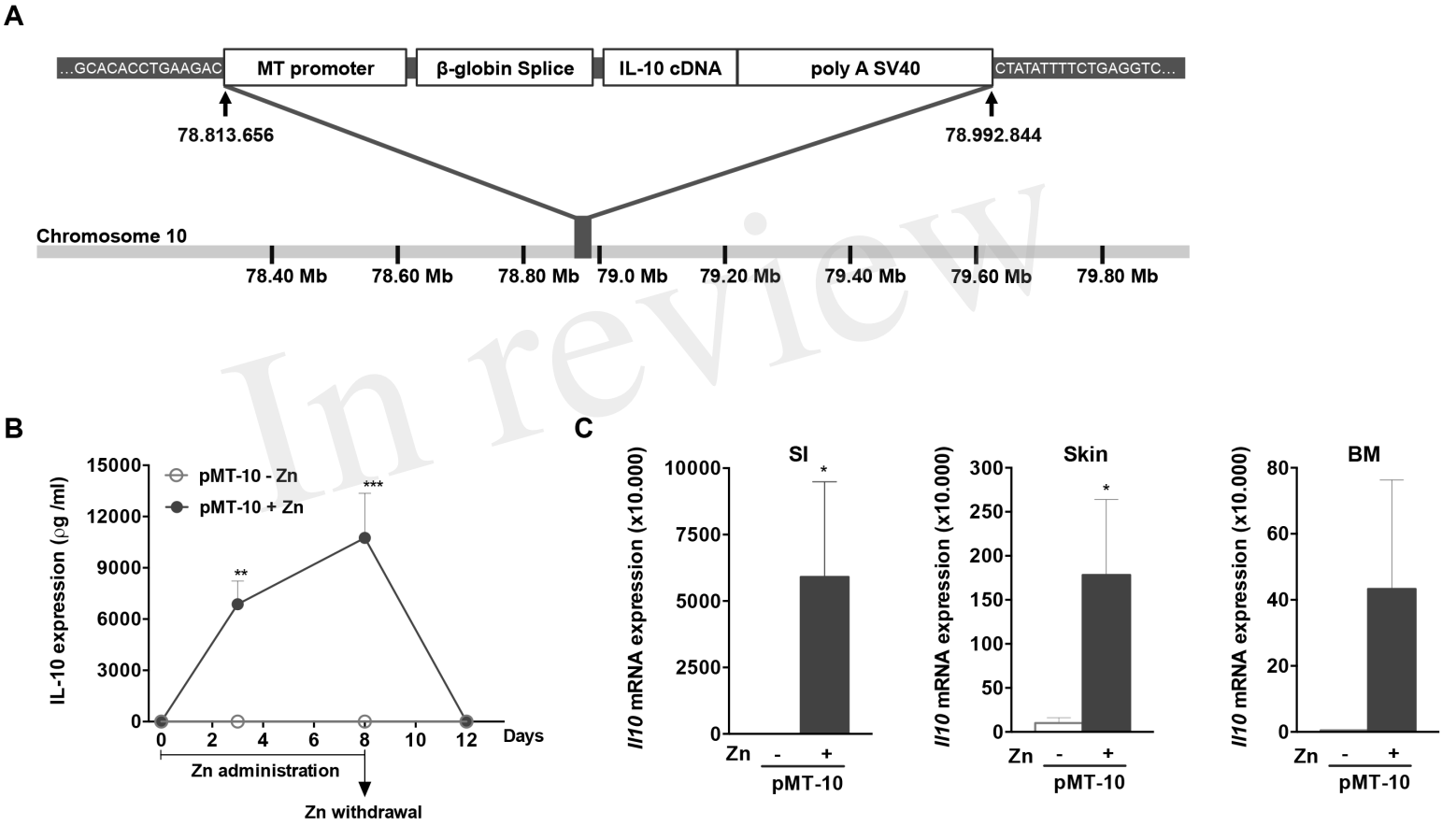

Figure 2.TIF

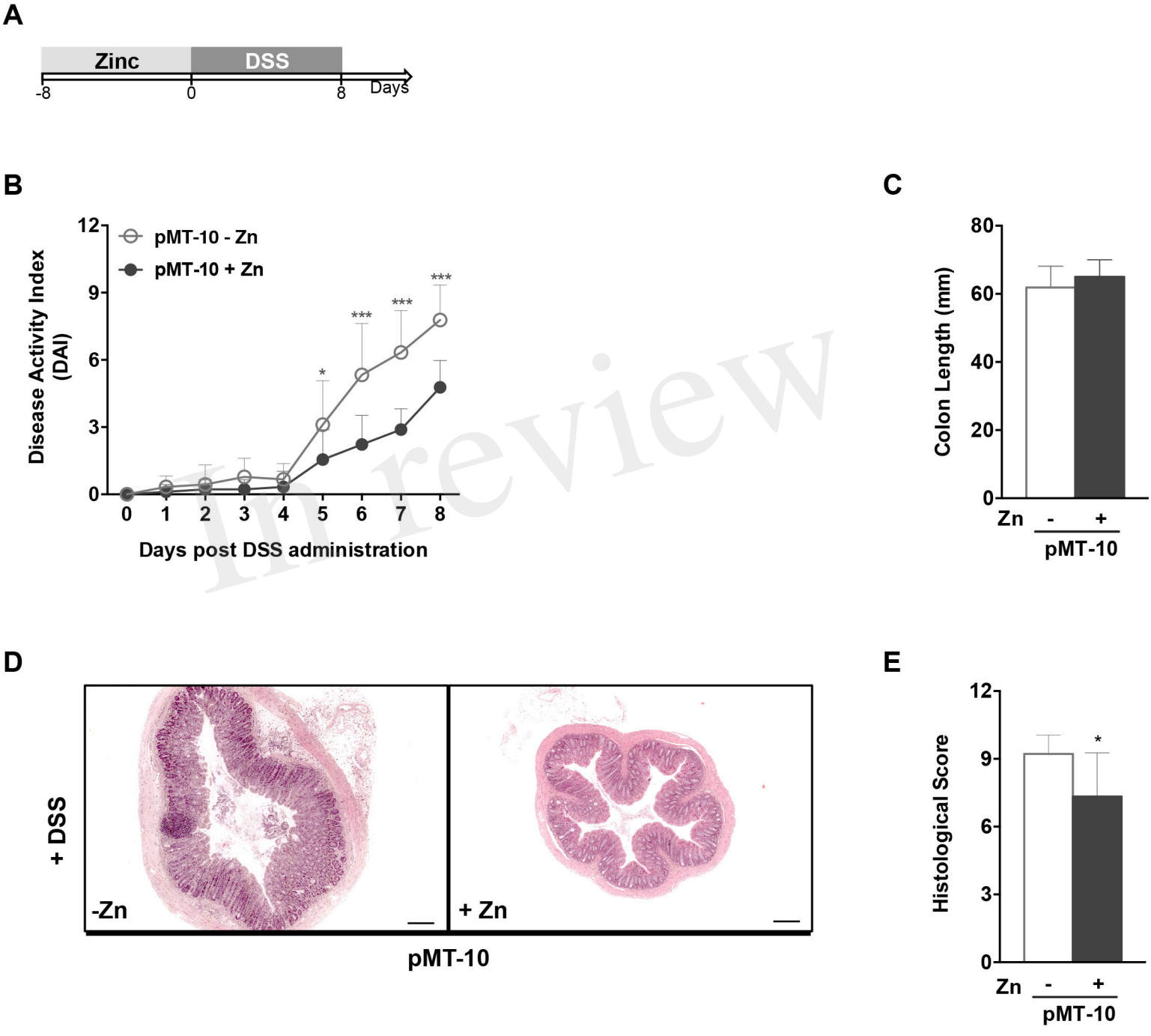

Figure 3.TIF

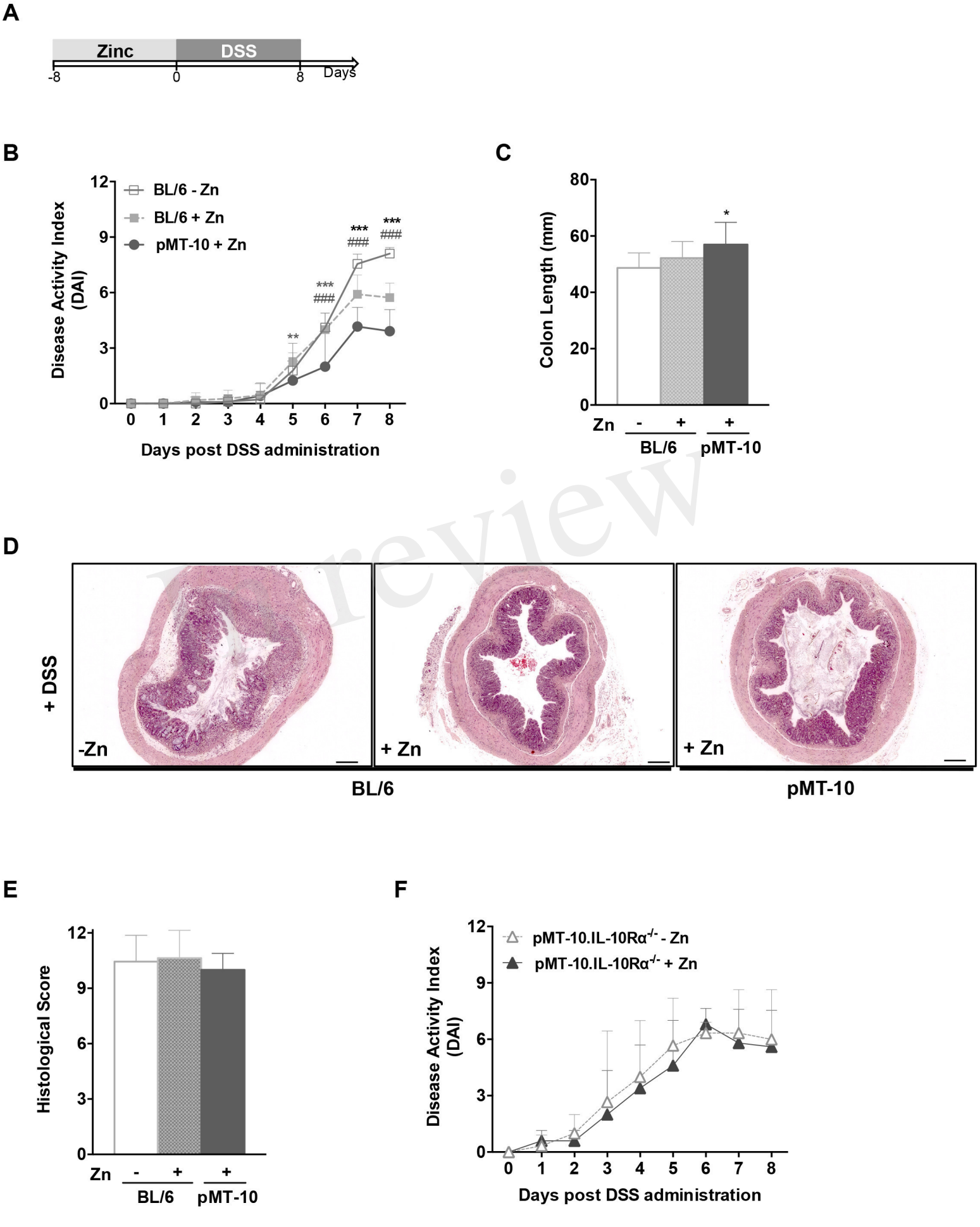
A
Zinc
DSS
-8
0
8
Days
B
Disease Activity Index (DAI)
BL/6 - Zn
BL/6 + Zn
pMT-10 + Zn
Days post DSS administration
C
Colon Length (mm)
Zn
BL/6
pMT-10
D
+ DSS
-Zn
+ Zn
+ Zn
BL/6
pMT-10
E
Histological Score
Zn
BL/6
pMT-10
F
Disease Activity Index (DAI)
pMT-10.IL-10Rα-/- - Zn
pMT-10.IL-10Rα-/- + Zn
Days post DSS administration

Figure 4.TIF

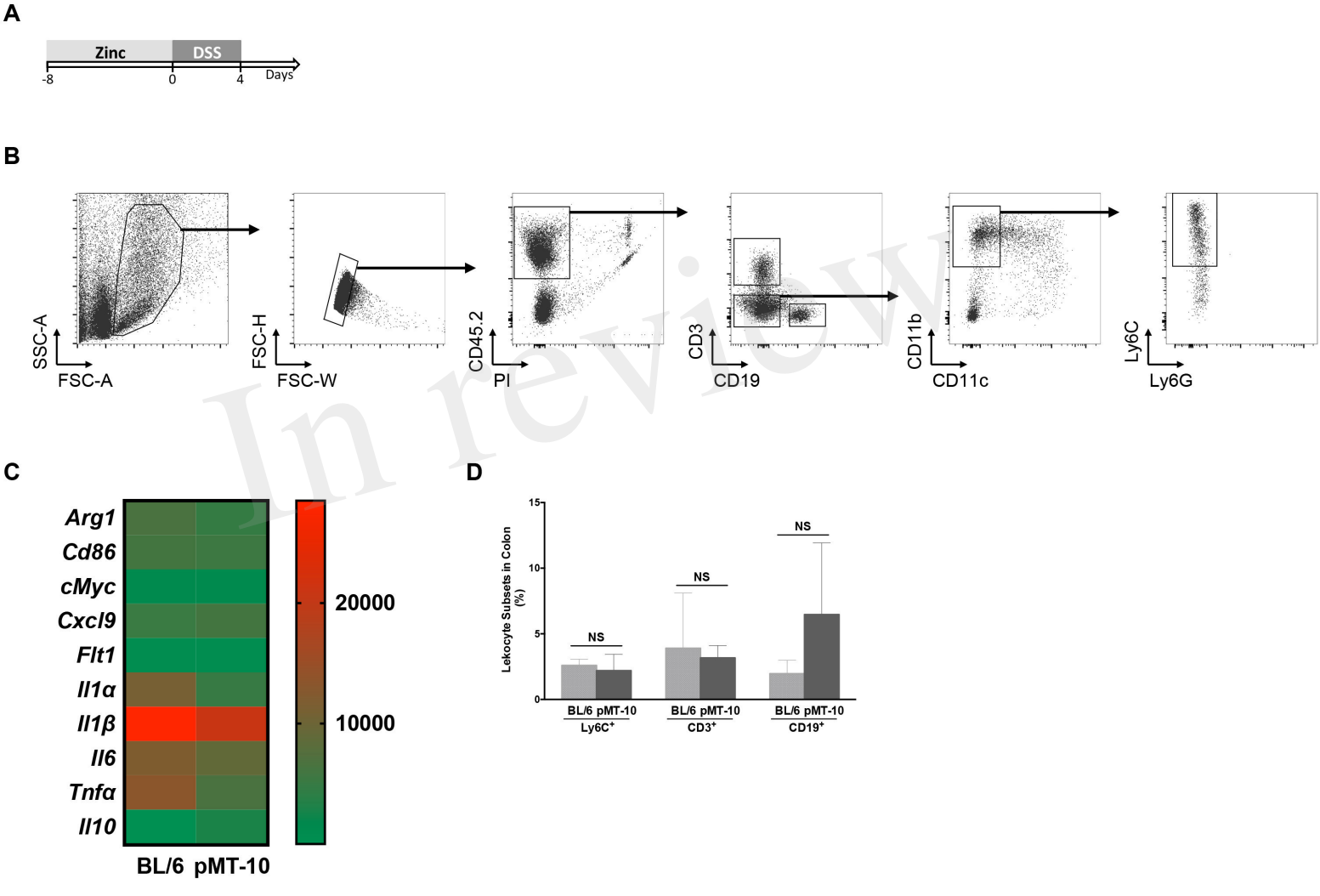

Figure 5.TIF