Interleukin-10 overexpression drives myeloproliferation and premature aging

Guilhermina A. Martins Carriche
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Orientador
Margarida Santos Saraiva, Ph.D., IBMC Porto

Coorientador
António Gil Castro, Ph.D., ICVS Braga
Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,
Porto, ______/______/_________
Let all of life be an unfettered howl.
Don’t stop to think, don’t interrupt the scream,
exhale, release life’s rapture.

Vladimir Nabokov
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<td>KLF1</td>
<td>Kruppel-like factor 1</td>
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<td>Lin−Sca-1^+\text{-}c-kit^+</td>
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<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<td>TET2</td>
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Resumo

A Interleucina 10 (IL-10) é uma citoquina anti-inflamatória que modela a resposta imunitária, sendo crucial para a homeostasia do sistema imune. A desregulação da sinalização da IL-10, e receptor da IL-10, está associada com inúmeras patologias, especificamente infeções crónicas e doenças carácter autoimune. Recentemente, o nosso laboratório situou a IL-10 como reguladora da hematopoiese. De facto, uma sobre-expressão de IL-10 conduz a uma aberrante mielopoiese, com a simultânea diminuição na limfopoiese, e ocorrência de hematopoiese extramedular. No entanto, os mecanismos moleculares pelos quais a IL-10 desregula e hematopoiese continuam indefinidos. Este projeto tinha como objectivo a investigação das vias moleculares que orquestram a mieloproliferação associada à IL-10. Recorrendo a um modelo animal que sobre-expressa IL-10 de uma forma indutível (o murganho pMT-10) e a uma série de ferramentas laboratoriais, que incluem inibidores químicos das vias moleculares, nós descobrimos que a desregulação na hematopoiese é sinalizada sobretudo via PI3K. Adicionalmente, propusemos que anómala mieloproliferação dirigida pela sobre-expressão de IL-10 dá origem a uma patologia semelhante à leucemia mielomonocítica crónica humana, uma doença frequentemente associada com o envelhecimento. Surpreendentemente, também observamos que uma contínua sobre-expressão de IL-10 promove o surgimento *in vivo* de características progeroides, culminando com a morte prematura dos murganhos. Estas observação levaram à hipótese da IL-10 estar a induzir senescência. Portanto, de forma a avaliar o possível papel para a IL-10 na senescência celular, nós estabelecemos culturas primárias de fibroblastos, estimuladas com IL-10 recombinante, e medimos a atividade de β-galactosidase – um marcador de senescência –, e a ativação de p21 – um marcador para a paragem do ciclo celular. Resultados preliminares indicam a IL-10 de facto como um indutor de senescência. Em conclusão, o trabalho apresentado nesta dissertação estabeleceu que a mieloproliferação induzida pela IL-10 é dependente da via molecular PI3K, apresentando também o pMT-10 como um possível modelo para indução de leucemia mielomonocítica crónica e envelhecimento prematuro.
1. Abstract

Interleukin (IL)-10 is an anti-inflammatory cytokine that functions as a modulator of the immune response, being thereby crucial for the homeostasis of the immune system. Deregulation of the IL-10/IL-10 receptor signaling has been associated with many pathologies, specifically autoimmune diseases and chronic infections. Recently, our lab placed IL-10 as a regulator of hematopoiesis. Indeed, an overexpression of IL-10 leads to aberrant myelopoiesis, with concomitant decrease in lymphopoiesis, and presence of extramedullary hematopoiesis. However, the molecular mechanisms by which IL-10 deregulates hematopoiesis remain elusive. This thesis aimed to investigate the molecular pathways orchestrating the IL-10-induced myeloproliferation. By resorting to the animal model for inducible IL-10 overexpression (the pMT-10 mouse) and to a series of tools, including chemical inhibitors of signaling pathways, we found that the deregulated hematopoiesis was mainly signaled via the PI3K pathway. Additionally, we propose that the observed IL-10-driven aberrant myelopoiesis gives rise to a pathology that resembles the human chronic myelomonocytic leukemia, a disease frequently associated with aging. Surprisingly, we also observed that a sustained IL-10 overexpression led to the in vivo emergence of progeroid features, culminating with the mice premature dead. Altogether, these observations led to the hypothesis that IL-10 might be inducing senescence. To evaluate a possible role for IL-10 in cellular senescence, we cultured mouse primary fibroblasts with recombinant IL-10 and measured the activity of β-galactosidase – a marker for senescence –, and the activation of p21 – a marker for cell cycle arrest. Preliminary data point to IL-10 as a senescence inducer, via cell cycle arrest. Future work will be performed to better explore the contribution of IL-10 to cellular senescence. In conclusion, the work presented in this thesis established that IL-10-induced myeloproliferation is dependent on PI3K signaling pathway, and assigned the pMT-10 as a possible model for immune-induced chronic myelomonocytic leukemia and premature aging.
2. Introduction

2.1. Hematopoiesis

Circulating blood cells are not only critical to replenish nutrients and oxygen to tissues, but also play a central role ensuring the organism immunity. Hematopoiesis is the process by which all blood cells are differentiated from hematopoietic stem cells (HSCs), throughout the lifetime of the individual. HSCs, a rare population residing in the bone marrow (BM), are able to self-renew, which denote them as long-lived (1). HSCs are also multipotent, proceeding to a hierarchical differentiation and thus giving rise to various blood cell populations, such as lymphocytes, granulocytes, monocytes, erythrocytes and platelets, which are mostly short-lived and need to be replenished (2). Importantly, defects along the hematopoietic process may lead to life-threatening diseases (3).

To understand the extent and complexity of hematopoiesis and cell fate commitment, the origin of the hematopoietic system needs to be considered. The production of blood cells starts in early stages during the embryonic development. It is believed that the yolk sac is the first site for hematopoiesis, followed by the aorta-gonad mesonephros region, the fetal liver, and finally, after birth, the BM (4). The first wave for blood production plays the main goal of generating red blood cells, in order to facilitate tissue oxygenation during embryo growth (5). Definitive hematopoiesis involves HSC migration and colonization of the fetal liver, thymus, spleen and finally BM (6). The timeline of events is inconsonant, since some authors argue that HSCs of the fetal liver circulate to the adult BM (7), and others observe that both fetal liver and BM are seeded during the same stage of development (8). Interactions between early lymphocytes entering the developing thymus – “thymic crosstalk” – shed some light on the possible role of myeloid and lymphoid progenitors in stimulating the growth of secondary hematopoietic sites (9). In conclusion, the establishment of a primitive immune system is thought to be achieved through a stepwise hematopoietic process, involving an already well-defined genetic program (10).
Hematopoiesis occurs in an orderly fashion in which HSCs generate progenitors and subsequently precursor cells, following a cellular fate commitment. The classical model for hematopoietic lineage branching is well established, where two major progenitor populations are defined: common lymphoid progenitors (CLPs), giving rise to precursors and mature B and T cells, and Natural Killer (NK) lineage cells; and common myeloid progenitors (CMPs), that generate megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) (11). MEPs further differentiate into megakaryocytes and erythrocytes, whereas GMPs generate neutrophils, eosinophils, basophils and monocytes (11). Figure 1 illustrates a simplified representation of the hematopoietic tree. Notably, lineage commitment is not static as there are numerous reports demonstrating that committed hematopoietic cells can be re-programmed and converted into cells of other lineages (12).

Figure 1. Representation of the hematopoietic tree. Long-term hematopoietic stem cells (LT-HSCs) have the potential for self-renewing and differentiating into more mature cells. In the BM, short-term (ST) HSCs differentiate into progenitor cells common lymphoid precursors (CLPs), common myeloid precursors (CMPs), granulocyte-monocytic precursors (GMPs) and megakaryocyte-erythrocyte precursors (MEPs), according to the organism requirements. HSCs and precursors are represented within the purple diagram. Mature hematopoietic cells, represented within the salmon diagram, are found throughout the circulatory system and within tissues.
Every cellular population of the hematopoietic tree is defined and characterized based on the expression of surface markers (1). The identification of HSCs from BM cellular suspensions is usually determined by the surface expression of Sca-1 and c-kit, within the Lin− fraction. Thus, HSCs are within the Lin−Sca−ckit+ (LSK) cellular population. Some authors further subdivide HSCs into long-term HSCs, short-term repopulating HSCs and multipotent progenitors (MPPs), being the latter a population without self-renewal ability, but with its differentiation potential still conserved (13). Typically, short-term HSCs and MPPs retain LSK markers, but additionally express FMS-like tyrosine kinase (Flt3) (14). Further into the hematopoietic tree, interleukin(IL)-7Rα is a characteristic marker expressed on CLPs and early B and T cells progenitors (15), whereas the distinctive marker for CMPs is CD34, which allows the distinction between GMPs, CMPs and MEPs. Mature hematopoietic cells also express characteristic surface markers; for instance, cells of myeloid nature are defined as CD11b positive (16), early and pro B cells both express CD19 (17), and T cells can be classified as CD4 or CD8 positive (18). As the hematopoietic tree starts to branch, the cellular pluripotency gives rise to the multipotency of the hematopoietic progenitors, from which the mature cells are differentiated. Eventually mature cells leave the BM and enter the circulating system (19). In contrast to all other lineages, that are exclusively BM-derived, T cells differentiate after migration of its progenitors into the thymus (20). After the maturation process, T lymphocytes also enter the bloodstream, and migrate to peripheral lymphoid organs.

The most abundant population of mature immune cells is of myeloid nature (21). Terminal populations of the myelopoietic tree such as macrophages, granulocytes and dendritic cells are of great importance due to their role in protecting the organism against pathogens, eliminating dying cells, and regulating tissue remodeling. During tumor progression, myeloid cells play functions associated with angiogenesis, metastasis and invasion (22). The differentiation state of a cell can be also defined by the tissue and microenvironment (23); for instance, tissue-resident macrophages are cells derived from monocytes that are in circulation in the peripheral blood, moreover, according to the microenvironment and organism requirements they can be polarized into M1, M2 or even tumor-associated macrophages (24). Of all granulocytes, the most ample population is the neutrophils; they have a potent microbicidal activity own
to their phagocytic capacity. In basal state, neutrophils are only released from the BM after conclusion of their maturation process (25); nevertheless, during infections, neutrophil precursors might be released in order to augment the organism response.

Because the differentiation of hematopoietic precursors into mature cells allows immune surveillance of the organism and efficient control of infections and inflammation, the regulation of the differentiation process needs to be tight and competent. Given that the main focus of this thesis is myelopoiesis, the upcoming sections will be centered on better describing this branch of the hematopoiesis.
2.2. Factors Regulating Hematopoiesis

Hematopoietic cell differentiation and commitment is a complex process mediated by several transcription factors, direct cell-cell interactions, environmental cues and cytokines (2). The impact of these elements can be instructive, thus directly affecting cell fate commitment and differentiation, or permissive, by supporting cell survival and/or expansion of progenitors already pre-committed. Of most relevance, transcriptional factors and cytokines are the better-studied regulators of hematopoiesis.

2.2.1. Transcription Factors

The processes leading to lymphoid or myeloid differentiation require deep changes in gene expression, and these are associated with regulatory networks of transcription factors (26).

Two well-known transcription factors for progenitor cells are Ikaros and Purine Rich Box-1 (PU.1). The Ikaros family of proteins is characterized by the presence of zinc finger motifs along their aminoacidic chain (27). These proteins orchestrate their role as hematopoietic regulators since early embryonic stages, and continue during adulthood (28). Mutations on several members of Ikaros family often result in hematological malignances, mainly of lymphoid nature, as mutated mice show a defective lymphopoiesis (29). PU.1 although also critical for the generation of progenitor B and T lymphocytes, is essential for myeloid cell differentiation and HSC maintenance (30). Its broad role was firstly attested by the observation that PU.1 null mice die at birth and display absence of myeloid and lymphoid cells (31).

Several transcription factors play a role in specific lineage commitment. Within the myeloid branch, CCAAT/enhancer-binding protein alpha (C/EBPα) is a key transcription factor for granulolytic-macrophage commitment. Indeed, mice defective for C/EBPα show insufficiency in neutrophils and eosinophils generation (32). It has been proposed that this factor acts in early stages of differentiation, since the deletion of C/EBPα by retroviral expression did not affect terminal differentiation of GMPs (33). Another important transcription factor is IFN regulatory factor 8 (IRF-8), which acts on the myeloid lineage by repressing the development of neutrophils and
promoting the emergence of dendritic cells (34). For the megakaryocytic-erythroid lineage, the most prominent transcription factors are GATA-1, Kruppel-like factor 1 (KLF1) and GFI1b (Growth factor independence 1). Indeed, deficiency in GATA-1 blocks the development of erythroid cells in embryos leading to lethal anemia (35); GATA-1 is also essential to the maturation of megakaryocytes. KLF1, being essential to erythropoiesis, is present in both precursor and definitive erythroid populations; similar to GATA-1, ablation of the KLF1-encoding gene leads to lethal anemia in embryos (36). Finally, GFI1b is expressed essentially in erythroid and megakaryocytic precursors (37), as in conditional GFI1b knockout mice the differentiation from pro-erythroblasts to mature erythroblast is substantially decreased (38). Of note, GFI plays a broad role in diseases such as neutropenia, myeloid leukemias or even allergies. In addition to myeloid promoting transcription factors, some of these molecules counter-balance myelopoiesis. This is for example the case for monocytic transcription factor MafB limits the ability of macrophage colony-stimulating factor (M-CSF) to induce myeloid commitment in HSCs (39).

These transcription factors do not work as sole elements. Indeed, lineage commitment and differentiation is an elaborate process and it requires an entangled network of components. For instance, the modulation of the myeloid-associated transcription factors depends on several cellular processes, such as those resulting from communication through cytokines.
2.2.2. Cytokines

Studies assessing the role of cytokines and cytokine receptors, on hematopoietic cells, suggest that these molecules likely play a permissive effect. This is for example illustrated by the observation that mice deficient for IL-2, IL-3 or IL-17. IL-2 – important for the regulation of the activation of T lymphocytes – plays a role in myelopoiesis, as mice with IL-2 deficiency develop anemia but maintain normal levels of peripheral blood leukocytes (40). Chen et al. studied the hematopoietic process in older mice defective for IL-2, and observed an overall hematological failure, with the development of anemia, lymphocytopenia, thrombocytopenia, splenomegaly, and thymus involution (41). IL-3 is a well-documented cytokine in contributing to differentiation of hematopoietic progenitor cells and maturation of myeloid cells (42). Moreover, IL-3 seems to be relevant for survival and proliferation of HSCs, a study from Robin et al. displayed the importance of IL-3 during the development of HSCs by amplifying and regulating the HSCs pool (43). IL-17 is also a potent mediator of hematopoietic responses, as it induces the production of relevant cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) (44). During in vivo overexpression of murine IL-17, the granulopoiesis lineage was stimulated, accompanied with a stimulation of G-CSF release (45). In brief, the broad role of IL-17 extends from its potential in expanding myeloid progenitors, to the initiation of the proliferation of mature neutrophils, and also stimulates the secretion of other hematopoietic-associated cytokines (46). Additionally to their active or permissive role in cell fate commitment, some cytokines hold the ability to induce lineage reprogramming, as it is observed for IL-2: experiments with IL-2Rβ-expressing CLPs in culture with medium containing IL-2 induced formation of GM colonies (47); similarly, also GM-CSFR expression in CLPs can dictate lineage reprograming, since stimulation with GM-CSF is observed to convert CLPs into myeloid cells (48).

Other cytokines appear to have an active function in the context of hematopoiesis. Genes encoding GM-CSF, G-CSF, or erythropoietin (EPO) evidenced a reduction in progenitor and mature myeloid cells, without a complete loss of the lineage (49). Indeed, in mice deficient for G-CSF, although peripheral blood neutrophils were considerably decreased, these cells were still present in the blood and BM (50), which indicates that G-CSF does not solely support neutrophil
production. Similarly, homozygous mice for a disrupted GM-CSF gene, showed that hematopoiesis did not suffer any major perturbation, with maintenance of normal levels of mature hematopoietic cells and their precursors in the blood, BM and spleen (51). Hong Wu et al. studied the role of EPO in the context of erythropoiesis, showing that in fact neither EPO, nor EPOR are required for erythroid lineage commitment of progenitors (52). Other studies report that indeed G-CSF, GM-CSF and EPO are important for hematopoietic cell function, but their absence is not detrimental to lineage commitment or differentiation (53, 54).

Apart from the meticulously studied cytokines cited above, others exhibit a role during the hematopoietic process. Of those, IL-6, IL-7, IL-15 and thrombopoietin stand out (42).

Importantly, the levels of cytokines vary during the life-time of an individual, for example during infection. Additionally, genetic mutations in cytokine or cytokine signaling pathways deregulate the cytokine response of a given cell. All this might impact the regulation of hematopoiesis, eventually leading to disease.
2.3. Deregulation of Hematopoiesis

Hematological cancers represent deregulations during the hematopoietic process, starting mainly in the BM, and affecting the production and function of blood cells (55). The hematological malignancies are diverse, presenting various categories of leukemia, lymphoma and myeloma (56). The cause for these blood cancers – as for the majority of solid neoplasms – is associated with errors of the DNA repair mechanism, resulting in spontaneous mutations (57). Nevertheless, cancers can be also caused by microbial agents, such as Epstein-Barr virus, or T-cell lymphocytotropic virus (58).

Myeloproliferative neoplasms (MPNs) are a group of hematologic diseases defined by an HSC-derived expansion of one or more myeloid cell lineages. According to the World Health Organization (WHO), MPNs are classified as: chronic myelogenous leukemia (CML), chronic neutrophilic leukemia, chronic eosinophilic leukemia, essential thrombocythemia (ET), mastocytosis, polycythemia vera (PV) and primary myelofibrosis (PMF) (59). Of these seven pathologies, three have been characterized as Philadelphia-chromosome-negative classic MPNs (60): PV, PMF and ET. In brief, patients with PV have an expanded erythroid lineage, consequently having a proneness to thrombosis, strokes, and hemorrhages (61). Individuals with PMF present a progressive marrow fibrosis, non-specific multi-lineage hematopoietic expansions, with occurrence of extramedullary hematopoiesis. PMF is the MPNs associated with worst patient outcome (62). ET is defined by proliferation of megakaryocytes and platelets in the BM and peripheral blood, leading to an increase incidence of thrombosis and bleeding (61). MPNs are of particular interest for this thesis, as the studied animal model presents several characteristics resembling MPNs, as discussed later on (section 2.6).

Genetic screenings unveiled the mutational landscape associated with several hematological neoplasms; as for instance, the FIP1L1-PDGFRα gene fusion in chronic eosinophilic leukemia; or BCR-ABL fusion gene with increased ABL tyrosine kinase activity in CML (63). Studies performed in the last decade showed that for the classic MPNs, the most relevant mutation to disease development is Janus kinase (JAK)2V617F mutation (64). This mutation is responsible for constitutively activate the JAK/Signal Transducer and Activator of Transcription (STAT) signaling pathway. An incidence of 97% of this mutation in PV and more than
50% for patients with ET and PMF was determined (60). Nonetheless, these percentages also indicate the possibility of other genetic factors and pathways being involved in the development of MPNs (61). In fact, more genetic mutations have then been associated with the disease, but the majority of the involved genes actually contribute to the epigenetic regulation of JAK2, thus affecting once more this pathway (65). For instance, mutations in Ten-Eleven-Translocation2 (TET2) – required for DNA hydroxymethylation – affect mostly HSCs and progenitors and are found in approximately 14% of MPN; RUNX1 mutations have been associated with the transformation of MPN to Acute Myeloid Leukemia (AML), contributing to the severity of the hematological disorder (66).

The importance of the JAK-STAT pathway in MPNs is reflected on the fact that the majority of target therapies available are classified as single-agent JAK inhibitors or combination of drugs with JAK-inhibitors (67). Ruxolitinib (Rx) is a JAK inhibitor agent FDA and EMA approved, and most prescribed drug to treat MPNs, associated with symptom improvement and reduction of the risk of death (68). Other JAK2 inhibitors are currently under clinical trials, nonetheless, due to the importance of this signaling pathway to a plethora of organism functions, several have been discontinued own to high toxicity (69).

In addition to alterations on the JAK-STAT signaling, as stated above, other molecular pathways have been studied. Of relevance is the phosphoinositide 3-kinase (PI3K) pathway. Aberrant signaling through PI3K pathway has already been extensively associated with many cancers, due to its role in cell growth and survival (70). Unveiling the association of PI3K with hematologic conditions allowed the discovery of several genetic mutations associated with increased risk of disease or decreased survival times (71). For instance, the constitutive activation of AKT and FOXO1 has been linked to a shorter survival rate of AML patients (72). Abnormal genetic programs leading to PI3K hyperactivation have been also observed in chronic myelogenous leukemia, acute lymphoblastic leukemia and non-hodgkin’s lymphoma (66). In MPNs, BM samples from patients showed an increased phosphorylation of AKT, which is indicative of the activation of PI3K pathway (73). Owing to the numerous reports on PI3K aberrant activation in hematologic conditions, a large number of inhibitors have been developed (74); nevertheless, currently there are no FDA-approved PI3K inhibitors for the treatment of MPNs.

To better understand the complexity of MPNs, and find possible therapeutic molecules, several animal models have been exploited. The majority of the mouse
models developed recapitulated the genetic background of MPNs. This is the case for retroviral transduction of BCR-ABL into murine BM cells; or of transgenic models to alter the expression level of a gene of interest, in an inductive or constitutive manner (75). Lacout et al. investigated the contribution of the JAK2 V617F mutation on MPNs using adoptive transfer of BM cells transduced with retrovirus expressing the mutation; the authors extensively studied the mice over-time and observed the development of features that mimic human PV (76). Aiming further, an interesting study by Tiedt et al. showed that the ration of mutant to wild-type JAK2 is able to alter the pathology of the MPN; the authors used an inducible Cre-loxP transgenic model, observing that induction of the mutation lower than the endogenous JAK2 orchestrate ET, whereas a higher ratio leads to PV (77). Apart from these mouse models for the direct study of MPNs, other animal models arose from the investigation of molecules not before associated with hematopoiesis. Mullenders et al. produced shRNA mouse model targeting molecules of the cohesin complex; they found that knockdown mice develop MPN, with occurrence of extramedullary hematopoiesis (78). Through the development of elegant animal models it is possible to decipher the molecules involved in the establishment and progression of MPNs, and additionally find therapeutic measures to alleviate disease symptoms.
2.4. Hematopoietic Senescence

The aging process of an organism occurs with the concomitant aging of its systems. Particularly, during aging the immune system starts to show a reduced capacity to respond to infectious agents, dysfunctional host cells, or in some cases higher incidence of cancers, such as MPNs (79). An aged immune system is characterized by a defective hematopoiesis, leading to deteriorations on the frequency and/or function of the blood cells produced. Thus the effects of immunesenescence are observed both in precursors and mature cells (80). Despite the notable evidences of alterations that immune cells undergo with aging, the process of immunosenescence is yet not fully understood, raising innumerous hypotheses.

Prevailing studies place the top of the hematopoietic tree – HSCs – as the starting point of the immunesenesce process (81). With age HSCs present particular characteristic changes. In an adult, HSCs are a population of low frequency, but both in aged mice and humans, a two- to tenfold increase in HSCs was found (82). With aging, the recruitment and mobilization of HSCs from the BM to the blood stream increases, hence higher percentages of HSCs in circulation are also observed (83). However, this expanded population appears to be functional defective, as studies show a reduced regenerative potential (84). Consequently, this dysfunction of HSCs results in reduced capacity to fully produce competent progenitors of myeloid and lymphoid lineages. In fact, aged HSCs present a skewed differentiation capacity towards myeloproliferation, with concomitant decreases along the lymphoid lineage (81), but with loss of functional competence. Moreover, As for T cells, thymus involution is a well-described phenomenon that accompanies aging, resulting in the defective generation of naïve T cells, thus decreasing the diversity in the T cell repertoire (85). As inferred before, in the BM of elderly people, an increased number of myeloid precursors cells is observed: nonetheless, these appear to develop into defective mature cells, as it is reported that both macrophages and neutrophils have reduced oxidative burst and phagocytic capacity (86).

Moreover, in individuals of advanced age a state of “inflammm-aging” is also detected, where elevated levels of soluble inflammatory immune mediators, such as IL-6, Tumor Necrosis Factor (TNF) and IL-1β are reported (87). It is believed that
these factors contribute for the progression of age-related inflammatory and neurodegenerative disorders (87).

The alterations impose by aging on hematopoiesis are illustrated in Figure 2.

Figure 2. Alterations imposed by aging in hematopoiesis. Aging of the hematopoietic process is reflected on the expansion of HSCs, shift towards myelopoiesis, and loss of functional competence of the mature immune cells. The blue scheme represents a young and healthy hematopoiesis, where HSCs display physiological potential for differentiation and self-renewal, giving rise to fully competent blood cells of lymphoid and myeloid origin. The grey scheme shows an aged hematopoiesis, with HSCs having an abnormal self-renewing capacity and skewed differentiation to myelopoiesis, generating mature cells functionally compromised.

The immunosenescence features cited above justify the susceptibility of aged individuals to develop diseases of hematologic nature (88). Indeed, the incidence for hematological neoplasms of myeloid nature increases greatly with age (89), being
the majority of patients with blood cancers older than 50 years. Besides the increased prevalence, older patients are also much more difficult to cure. Data show that for patients older than 65 years, the 5-year survival rate for lymphoma is about 50% and for myeloma is 20% (56).
2.5. Interleukin-10: a Crucial Immune Regulator

IL-10 was firstly described as cytokine synthesis inhibitory factor (CSFI), as it was indicated as a product of T-helper 2 (Th2) cells that inhibited proliferation and effector function of T-helper 1 (Th1) cells (90). IL-10 was readily identified as an inhibitor not only of IFN-γ synthesis by Th1 cells, but also of other known inflammatory cytokines, such as IL-2, IL-3, and TNF. Subsequently, other interesting observations were made. Malefyt et al. studied the production of IL-10 by monocytes upon LPS stimulation, demonstrating that its production occurs later when compared to other cytokines and that IL-10 reduces the expression of class II major histocompatibility complex (MHCII) (91). Other studies emerged where the inhibitory role of IL-10 on immune cells hampering cytokine secretion, NO production and MHC expression was reported (92, 93). All these results expose the regulatory role of IL-10 in controlling immune cell functions. IL-10 is now seen as a critical anti-inflammatory cytokine, playing an important part in establishing tissue integrity homeostasis, by restraining inflammatory responses, thus preventing collateral damage during the immune response (94).

2.5.1. IL-10 and the IL-10 Receptor

IL-10 acts by binding to its surface heterodimeric membrane receptor composed of a α and a β chain: the IL-10 receptor (IL-10R) (95). The ligand-binding subunit is the IL-10Rα; it is expressed in hematopoietic cells, but with variable levels of expression (96). The interaction between the protein IL-10 and its receptor leads to signal transduction pathways, via the JAK/STAT system (97). Upon IL-10 binding, the interaction between IL-10/IL-10Rα adopts conformational changes in order to allow association with IL-10Rβ. The IL-10Rβ is not a major supporter for the IL-10 binding, instead its main function is to recruit JAK into the signaling complex (98). JAK1 and Tyrosine Kinase 2 (Tyk2) are then activated promoting the phosphorylation of the IL-10Rα chain on two tyrosine residues, causing the activation of transcription factors of the STAT family (99). Of most importance, STAT3 is essential for IL-10 signaling;
studies claim the exclusivity of STAT3 in signaling the anti-inflammatory effects of IL-10 (100). Nevertheless, as these conclusions were not drawn for all cells of hematopoietic origin, the involvement of more proteins of the STAT family is possible. In support of this, a study found that JAK2 inhibitors might be effective in treating diffuse large B-cell lymphoma, since the lymphoma cells present a constitutive activation of STAT3, due to signaling via JAK2 (101).

Recently, another signaling pathway for IL-10 responsiveness was unveiled: the PI3K-AKT-GSK3 pathway (102). The PI3K-AKT-GSK3 pathway is associated with several cellular functions, including proliferation, differentiation, metabolism, growth, and survival (103). Jihong Shi and colleagues propose that the anti-fibrotic potential of IL-10 on fibroblasts is mediated via synergistic activation of PI3K-AKT and STAT3 (104). In another study, T. T. Antoniv and L. B. Ivashkiv explored the role of PI3K signaling in response to IL-10; their findings on human macrophages stimulated with lipopolysaccharide show that PI3K was able to increase IL-10-mediated inhibition of induced expression of IL-1, IL-8 and cyclo-oxygenase-2 (102). Gunzl et al. used a model for constitutively active PI3K in macrophages, and observed that cells adopted a strong anti-inflammatory profile via up-regulation of IL-10 (105). These studies establish and accentuate the relevance of PI3K signaling cascade on the response to IL-10 and IL-10 action.

Besides its clear immunologic role in cytokine signaling, STAT3 has been also associated with cancer, since many solid tumors appear to express excessive levels of STAT3 (99). Kortylewski et al. found that inhibition of STAT3 in tumor-bearing mice improves the immune surveillance system, contributing for the inhibition of tumor growth and metastasis (101). These observations made for STAT3 are in agreement with the puzzling role of IL-10 in cancer biology, as mice and humans with deficits in IL-10 signaling appear to develop tumors more easily and rapidly (106). Indeed, IL-10 has been associated with the stimulation of cytotoxicity of tumor-resident CD8+ T cells, being observed a decrease of CD8+ T cells in tumors developed in IL-10−/− mice (106). However, given the major role of IL-10 as an anti-inflammatory and immunosuppressive cytokine, IL-10 is found to be produced by some tumor cells, such as lung cancer, melanoma or lymphoma, in order to induce the down-regulation of major histocompatibility complex class II expression in antigen presenting cells (107). In all, IL-10 appears to have a controversial role in cancer immunology, supporting the need for a fine balance of IL-10 anti-inflammatory role. Moreover, IL-10 and IL-10 signaling are critical in the gut homeostasis, with mice
lacking IL-10 or the IL-10R developing spontaneous colitis (108). Therefore, failing of the IL-10-IL-10R system has detrimental and potentially life threatening consequences.
2.5.2. IL-10 and Hematopoiesis

The importance of IL-10 to immune homeostasis, as a potent anti-inflammatory cytokine and regulator of inflammation, is undeniable (94). A possible role for this cytokine in hematopoiesis remains unknown, but several pieces of evidence hint to it. Indeed, LSKs express the surface receptor for IL-10 (109), attesting their ability to respond to IL-10. Additionally, HSCs stimulation with IL-10 enhanced their self-renewal potential (110). The authors took advantage of an IL-10 deficient mouse model and ex vivo cultures, observing a decrease of progenitor hematopoietic populations in the BM of IL-10−/− mice; an enhanced HSC regeneration in stromal cultures that produce IL-10; and lastly, the induction of HSC self-renewal in purified LSK cultures stimulated with exogenous IL-10 (110). Based on these observations, the authors raised the possibility of IL-10 as a cytokine bridging the immune and hematopoietic systems. Another ex vivo study showed a pronounced augmentation of myeloid progenitor cells when CD34+ cells cultured with a standard cocktail for cell survival were supplemented with recombinant IL-10 (111). However, other lineage-associated cytokines were present in the cocktail used as culture medium, being the possible effect of IL-10 on lineage-commitment possibly masked.

Oehler et al. studied the effect of IL-10 on spontaneous hematopoietic colony formation in normal human peripheral blood mononuclear cells (PBMCs). Their findings place IL-10 as a regulator of hematopoiesis by mediating the release of GM-CSF by accessory cells (112); additionally, they conclude that IL-10 suppresses spontaneous myeloid colony formation by PBMCs, and that this suppressive potential is restored upon the addition of GM-CSF (112). Both studies seem to be contradictory, but the variability of the results might be due to poor and obsolete cell isolation techniques, which can cause culture contamination with cells and/or molecules of relevance to hematopoiesis. Other reports evaluating the role of cytokines in hematopoiesis have positioned IL-10 as an indirect modulator of this process. Fine et al. demonstrated that exposure of cells to IL-10 and IL-7 prompted an enhancement in murine pre-B cell colony formation, in spite of IL-10 by itself being incapable of any effect on lymphopoiesis (113). In another study, by Rennick et al., the differentiation potential of IL-10 was measured by colony-forming assays, having the authors concluded that IL-10 solely does not lead to the generation of any mature cell; yet when in combination with other cytokines – such as IL3, IL-6, EPO or IL-11 –
the growth of megakaryocyte colonies was observed (114). IL-10 was also showed to act synergistically with EPO enhancing CFU-Erythroid growth (115). All these reports fail to provide a specific role for IL-10 in lineage-commitment, since the presence of other cytokines was always required. Moreover, the overall action of IL-10 in lineage commitment, how IL-10 might regulate the hematopoietic system, or the molecular pathways involved in IL-10-induced HSCs self-renewal remains unknown.

The in vitro studies performed on the role of IL-10 in hematopoiesis are summarized in Figure 3.

![Figure 3. In vitro studies establishing a role for IL-10 in hematopoiesis display contradictory findings. Several studies suggest the relevance of IL-10 in regulating proliferation and differentiation of hematopoietic cells. Nonetheless, a clear role for IL-10 has not been established, as a differentiation profile is only obtained in the presence of other cytokines. Additionally, some reports present contradictory conclusions. With each finding is associated the correspondent reference. Arrows indicate enhancement (↑) or inhibition (↓) of a given process.]

The importance of IL-10 and its clear multidisciplinary promoted the progression of in vitro studies to its translation into in vivo clinic studies. Sosman and
his team conducted a randomized double-blinded and placebo-controlled clinical trial to assess the ability of IL-10 to treat thrombocytopenia. To do so, they administered recombinant IL-10 in 12 healthy subjects. A significant decrease on platelet production was reported, independent of thrombopoietin (TPO) effects, since the levels of TPO in the serum of the patients did not decrease (116). Additionally, the authors suggest that IL-10 is an appellative candidate for the treatment of myeloproliferative conditions, such as PV, where an overproduction of progenitor cells is observed (116). Besides being quoted as a therapeutic molecule for myeloproliferative diseases, IL-10 was also studied in the context of aplastic anemia: Asano et al. administered recombinant IL-10 to seven patients with aplastic anemia, reporting a twofold increase of CFU-Erythroid formation in the presence of 10ng/mL of IL-10 in two of the seven cases examined (117). In spite of the unsatisfactory results, this was the first clinical study where the potential therapeutic effect of IL-10 on aplastic anemia was investigated. However, the small number of experimental subjects restrains the applicability of the results.

In conclusion, all previous studies assign a possible role for IL-10 during the hematopoietic process, observing differences in cell fate commitment; yet they all failed to specifically characterize this role, or to identify possible molecular pathways for IL-10 action.
2.6. The pMT-10 Mouse Model

The mouse model explored during this thesis was the pMT-10, an animal model generated by Drs. P Vieira and AG Castro. The pMT-10 mice were generated on a C57BL/6 background and are a model for zinc-inducible production of IL-10. In brief, the mouse IL-10 cDNA was cloned into a p169ZT vector, which carries a sheep metallothioneine (MT) Ia promoter (118). The resulting vector – pMT-IL10 – was injected in C57BL/6 eggs and transgenic founders were identified by PCR using primers for the IL-10 sequence and MT promoter. Upon administration of zinc, the pMT-10 mice display overexpression of IL-10 within 2 days after induction.

Considering the controversial data on the impact of IL-10 on hematopoiesis, its key function in the immune system and the fact that IL-10 is produced during the immune response, with many cells responding to it, we set on the study of IL-10 in hematopoiesis. For this, pMT10 mouse model is being studied in our laboratory. Unpublished data by Ana Cardoso et al. reveals deeper insights to the action of IL-10 on the hematopoietic process. The study revealed that an overexpression of IL-10 affects the hematopoietic tree resulting in clear alterations in various hematological parameters, compatible with myeloproliferation. Mice overexpressing IL-10 have increased numbers of myeloid cells, with decrease on erythrocytes, platelets and B cells, which denotes the imprinted tendency for myeloid lineage commitment. Furthermore, splenic alterations were also observed, namely marked splenomegaly, histological disorganization, and extramedullary hematopoiesis. Finally, preliminary experiments show that IL-10 overexpression led to the premature death of the animals. The overall characteristics observed in pMT-10 mice over-expressing IL-10 mimic those observed in other mouse models and in patients of MPNs.
2.7. Aims

As mentioned before, previous work done at our lab established that IL-10 contributes to a deregulation of the hematopoietic process. Since the molecular pathways triggered by IL-10 and the cause of death of pMT-10 mice over-expressing IL-10 remain to be studied, this work aimed to:

I. Establish a timeline of events between the BM and spleen, for the action of IL-10 in promoting myeloproliferation.

II. Understanding the signaling cascades that might be involved and their significance for clinical purposes.

III. Decipher the possible role of IL-10 during hematopoietic senescence, and the cause of death upon IL-10 overexpression.
3. Methods and Materials

3.1. Animal Model

Throughout this project, induced pMT-10, non-induced pMT-10 and C57BL/6 mice were used, with ages between 7 and 10 weeks old. Mice were housed at a conventional animal facility, with controlled temperature and humidity, and given food and water ad libitum. All mice procedures were performed in accordance with the general guidelines for animal welfare and ethic animal treatment: European union directive 11686/609/EEC and previously approved by the national authority “Direção Geral de Alimentação Veterinária”.

3.2. IL-10 Induction in pMT-10 mice

IL-10 overexpression was induced via administration of zinc sulfate heptahydrate (Sigma-Aldrich, USA). A solution of 50nM zinc with 2% sucrose was prepared in the drinking water and was delivered to the mice ad libitum, during the experimental period. The zinc activates the MT promoter, and within 2 days IL-10 reaches its maximum serum expression of 20ng/mL.

3.3. Buparlisib and Ruxolitinib Administration

Buparlisib (NVP-BKM120, Novartis, Basel, Switzerland) – a PI3K inhibitor – and Ruxolitinib (NVP-CCA0022, Novartis, Basel, Switzerland) – a JAK1/JAK2 inhibitor – were used to evaluate the signaling pathways activated during IL-10 overexpression. The administration of Buparlisib (Bp) and Ruxolitinib (Rx) was performed by oral gavage, once daily, during the experimental period. Bp and Rx concentration was determined based on previous reports using the mice as animal model. The inhibitors were freshly prepared, every two days, in methylcellulose solution. The methylcellulose solution alone was used on the control groups.
3.4. BM and Spleen Cell Suspensions

Mice were humanely euthanized by CO₂ inhalation. Blood was collected by heart puncture. Mice were washed with 70% ethanol and a cut was made in the fur, near the abdominal region, with a scalpel. The fur was removed to expose the forelimbs and the abdominal cavity. Femurs, tibias and spleens were recovered and conserved on complete medium HBSS (Hank’s Based Salt Solution, Gibco) supplemented with 10% Fetal bovine serum (FBS). BM cells were harvested from femurs and tibias with aid of 26G needle and syringe, by flushing with HBSS complete medium. The spleens were mechanically disrupted, by mashing the tissue with a 40um cell strainer, in a recipient containing HBSS complete medium. Cell suspensions were then centrifuged at 1300rpm for 7 minutes at 4°C. Depending on the experiment aim, the erythrocytes were lysed using erythrocyte lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA in dH₂O) incubated for 5 minutes at room temperature; or resuspended in the HBSS complete medium, when the exclusion of erythrocytes was not required. Following the lysis of erythrocytes, cells were washed by adding complete medium and centrifuging at 1300rpm for 7 minutes, at 4°C. After, cells suspensions were resuspended in fresh media for posterior analysis. Total numbers of cells were determined using an automatic cell counter (Countess, Invitrogen, USA).

3.5. Flow Cytometry

Cells from BM and spleen were stained for hematopoietic progenitors and mature subsets. To enrich the hematopoietic progenitors subset, first, a depletion of mature populations was performed. A cocktail for the mature subsets was prepared using anti-CD3, anti-CD4, anti-CD8, anti-CD11c, anti-CD11b, anti-CD19, B220, anti-NK1.1, anti-GR1 and anti-TER119, all in biotin. BM and Spleen cells were incubated with the biotin-antibody cocktail for 20 minutes at 4°C, and washed twice with complete medium by centrifuging at 1300rpm for 7 minutes. Cell suspensions were then incubated with Anti-Biotin MicroBeads (MACS, Miltenyi Biotec, Germany) for 15-20 minutes, at 4°C. Proceeding a final washing step, cells were ready to magnetic separations with LS Columns (MACS, Miltenyi Biotec, Germany). Briefly, columns
were carefully placed in the magnetic field of the MACS Separator. All columns were pre-washed with complete medium for higher recover. Cells are distributed along the columns; cells that pass through, without getting attached to the magnetic field, are the unlabeled cells, and represent the enriched lineage negative population, the hematopoietic progenitors. After collecting the negative cell fraction, a final washing step washing step was done by centrifuging for 7 minutes at 1300 rpm. Cells were then counted with an automatic cell counter (Countess, Invitrogen, USA), and stained with progenitor markers, namely: anti-FcgR PE; anti-CD34 APC; anti-cKit APC_Cy7 and anti-SCA-1 FITC, for 20 minutes at 4°C. Streptavidin (SAV) was also used to verify the sample purity.

The mature populations were assessed by directly incubating BM and spleen cells with anti-CD11b PE; anti-Ly6C PBlue; anti-Siglec-F APC; anti-CD11c PE_Cy7 and anti-Ly6G APC_Cy7, for 20 minutes at 4°C.

Stained cells were acquired and analyzed either with LSRII or FACSCanto II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Data were analyzed in FlowJo software (Tree Star, Ashland, CA, USA), using the gating strategies presented in Figure 4.

Figure 4. Flow cytometry gating strategy to identify LSKs, and progenitor populations CMPs, GMPs and MEPs. Shown are representative dot plots indicating the gating strategy to identify the cellular populations of control pMT-10 (top figure) and induced pMT-10 (bottom figure), firstly isolating single cells, followed by the lineage negative cells, and finally the LSKs and myeloid progenitors. The antibodies used are as indicated in the Materials and Methods. The numbers represent the % of cells within each population.
3.6. Colorimetric Staining for Tissue Characterization

3.6.1. Sample Preparation

To prepare samples for histological analysis, spleen, gut, liver, and skin samples were fixated in 10% formalin for 48 hours and then embedded in paraffin blocks. Tissue was cut in 5µm slices, deparaffinized and rehydrated for further hematoxylin & eosin (H&E), and Perls’ staining for liver sections.

3.6.2. Hematoxylin & Eosin

For the overall examination of the structure of tissues, it was performed Hematoxylin & Eosin staining. Firstly, slides containing the tissue preserved in paraffin are deparaffinized and rehydrated as it follows: submersion three times in xylene (Fischer Scientific, UK), 10 minutes each; followed by sequential submersion in 100% ethanol (EtOH), during 10 minutes, 95% and 80% EtOH for 5 minutes. Finally sections are washed in deionized H₂O and stained with hematoxylin solution (Sigma-Aldrich, MO, USA) during 45 seconds, being then rinsed with deionized water. To destain, the sections were rapidly dip in acid ethanol and washed again. Samples were immersed in Eosion solution (Sigma-Aldrich, MO, USA) for 30 seconds. To dehydratate, slides were successively dipped for 5 minutes in 95% and 100% EtOH, followed by 30 minutes of 15% in xylene.

3.6.3. Perls Prussian Blue Staining

To evaluate the deposition of iron in the liver, it was performed a Perls Prussian Blue Staining. Liver sections previously cut were placed two times in xylol (Fischer Scientific, UK), 10 minutes each time. The slides were then treated with gradient percentages of EtOH: 10 minutes in 100% EtOH; 5 minutes in 96% EtOH; 5 minutes in 70% EtOH; 5 minutes 50% EtOH. Afterwards, slides were washed in dH₂O 3 times, during 5 minutes each time. Freshly prepared Perl's solution (filtered solution of 2% of Potassium hexacyanoferrate (II) trihydrate (Merck) and 2%
hydrochloric acid 37% (Sigma-Aldrich, MO, USA) in deionized H₂O) was made. The slides were stained with Perl’s solution for 30 minutes, followed by a washing step with deionized H₂O during 5 minutes. The, slides were stained for 1 minute with Neutral Red (Sigma-Aldrich, MO, USA), and washed again with dH₂O water. To dehydrate, slides were rapidly and successively submersed in 70% EtOH, 96% EtOH, 100% EtOH; and 10 minutes in xylol. Finally, slides were mounted in Entellan (Sigma-Aldrich, MO, USA).

3.7. Blood Smears

Blood smears were stained with Wright stain solution (Sigma-Aldrich). A drop of blood freshly collected from each animal was applied in an extremity of a glass slide (Menzel-Glaser, Thermo Scientific). With the aid of other slide, the drop was spread along the initial slide, and let to dry. Submersion of samples in Wright solution was performed for approximately 15 seconds. After, slides were washed with dH₂O.

3.8. Fibroblasts Cell Culture

A primary cell culture of fibroblasts were established by mechanically and enzymatically digest ears of adult C57BL/6 mice. After harvesting, the ears were washed with PBS, and placed in DMEM:F12 with 0.125mg/mL collagenase IIS (Sigma) and 1.25mg/mL collagenase type II (Gibco). The ears were then cut in small pieces, with the aid of tweezers and surgical scissors. The specimens were incubated for 45 minutes at 37ºC and 5% CO₂. Enzymatic digestion was stopped by adding DMEM:F12 medium supplemented with 10% FBS, 1% antimycotic antibiotics.

The cell culture was kept at 37ºC and 5% CO₂. Possible tissue lumps were removed by washing with PBS 1x and suction needle. The medium was replaced every three days. Once adherent fibroblast reached confluence, cells were transferred to a 25cm² flask.
3.9. Senescence and proliferation

3.9.1. Cell culture

Fibroblasts were firstly grown on a 24-well plate, onto sterilized glass coverslips coated with fibronectin (Sigma-Aldrich, MO, USA), in DMEM:F12 supplemented with 10% FBS and 1% antibiotic-antimycotic solution. The culture was kept in an incubator at 37°C with 5% CO₂. Cells were then stimulated with varying doses of recombinant IL-10 (R&D Systems), namely, 10ng/mL and 50ng/mL, during different time periods, 24 and 48 hours. Control cells were not stimulated with recombinant IL-10.

3.9.2. Senescence-associated β-galactosidase activity

Cells previously cultured on sterilized glass coverslips, were submitted to senescence-associated β-galactosidase assay by incubating with 100nM bafilomycin A1 (Sigma-Aldrich, MO, USA) for 90 minutes at 37°C, 5% CO₂. Then 33µM of 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG, Sigma-Aldrich, MO, USA) was added to the cell culture and cells were again incubated for 90 minutes. The cells were fixed in freshly prepared 4% paraformaldehyde in PBS for 15 minutes at room temperature, washed with PBS three times and then permeabilized, for 15 minutes at room temperature, in PBS buffer containing 0.1% Triton-X 100. After fixation and permeabilization steps, cells were washed in PBS-T (PBS with 0.05% Tween 20). Slides were washed and counterstained with DAPI (1 µg/mL) (Sigma-Aldrich, MO, USA).

3.9.3. Ki67 and p21 staining for proliferation and cell cycle arrest

For immunostaining, cells formerly cultured onto sterilized coverslips, were fixed in freshly prepared 2% paraformaldehyde in PBS for 20 minutes at room temperature, washed with PBS three times and then permeabilized for 7 minutes at
room temperature in PBS buffer containing 0.3% Triton-X 100. Following fixation and permeabilization, cells were washed in PBS-T three times, and blocked with a solution of 10% FBS in PBS-T for 1 hour at room temperature. Cells were then incubated at 4°C overnight with primary antibodies dilution in 5% FBS in PBS. Later, cells were washed in PBS-T three times, and incubated at room temperature for 45 minutes with secondary antibodies diluted in 5% FBS. Cells were finally washed, stained with DAPI for 10 minutes, and the coverslips were mounted on microscope slides in an anti-fade solution containing 90% glycerol and 0.5% N-propylgallate. Primary antibodies were diluted as follows: mouse anti-p21 (SC-6246, Santa Cruz Biotechnology, CA, USA) 1:1000; mouse anti-ki67 (M-19, Santa Cruz Biotechnology, CA, USA) 1:1000. Secondary antibodies were diluted as follows: Alexa Fluor 488 and 568 diluted 1:1500 (Life Technologies CA, USA) and DNA was counterstained with DAPI (1µg/mL) (Sigma-Aldrich, MO, USA).
4. Results and Discussion

4.1. Molecular Mechanisms Underlying IL-10-induced Myeloproliferation

As stated before, pMT-10 mice induced to over-express IL-10 develop a phenotype of pronounced myeloproliferation. In the first part of this thesis, we focused in obtaining a more detailed picture of the IL-10-induced hematopoietic deregulation and of the involved molecular players and signaling cascades.

4.1.1. IL-10-induced Myelopoiesis Occurs in the Bone Marrow and Spleen at Different Time Points

The phenotype observed on induced pMT-10 mice affects the overall hematopoietic process, with notorious physiologic alterations, such as abnormal hematological parameters, with the propensity to a myeloproliferation, the occurrence of extramedullary hematopoiesis, and splenomegaly. Previous and current studies performed in our lab placed the BM and the spleen as cardinal organs responding to the IL-10-induced overexpression phenotype. Nonetheless, the initial target for the action of IL-10 remains unclear. To answer this question a kinetic assay was performed. pMT-10 mice were fed with zinc in water for 7, 15 and 30 days. At each time point, serum from the blood was collected and cell populations from the BM and spleen analyzed by flow cytometry. Non-induced pMT-10 mice were used as control groups. Figure 4 shows the gating strategy used to identify the different cellular populations under study: LSKs, myeloid progenitors and mature myeloid cells. At day 7 post-IL-10 induction, a significant increase of LSKs and GMPs in the BM, accompanied with decrease in CMPs, is observed remaining at a relatively constant level after that time-point (Figure 5). The spleen follows a kinetic pattern similar to that of the BM, with a substantial increase in LSKs visible already at day 7. However, the populations of GMP and CMP are affected in the spleen at a later phase than in the BM. The fact that, in the spleen, the increase of GMPs occurs later, might suggest that this population requires a stage of cell migration from the BM, preceding
IL-10 over-expression drives myelopoiesis and premature aging

a period of expansion and differentiation in the spleen. This hypothesis is further supported by the spleen size assessment shown in Figure 6, since the splenomegaly – well evident on day 30 of IL-10 overexpression by a significant increase in spleen weight – is not visible on day 7 on IL-10 induction. The striking decrease of CMPs is noticeable and somehow surprising, consider the increase of GMPs. A possible explanation is that this population is being quickly exhausted owing to the abnormal requirement for GMP production. The population of MEPs presented no significant alterations when comparing control and zinc-induced pMT-10 (data not shown).

A time-dependent increase in mature cell populations, precisely CD11b^+Ly6C^+Ly6G^- and CD11b^+Ly6C^-Ly6G^+, was also observed in both the BM and the spleen (Figure 7). This suggests that the expanded GMP population is likely competent in both organs, being able to differentiate into mature myeloid cells. Together, the combined increase of progenitors and mature cells most likely accounts for the observed splenomegaly. Moreover, the increase of GMP in the spleen hints at the occurrence of extramedullary hematopoiesis.
Figure 5. Percentage of LSKs and hematopoietic progenitor cells over time in the bone marrow or spleen of control and induced pMT-10 mice. The percentage of LSKs, CMPs and GMPs was measured by flow cytometry at days 0, 7, 15 and 30, after zinc-induced IL-10 overexpression. Induced pMT-10 mice show an increased percentage of LSKs and GMPs both in BM (left panel) and spleen (right panel), with concomitant decrease of CMPs. The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; *** represent a p value ≤ 0.001; One-way ANOVA test. SEM, standard error of the mean. Data are from one representative experiment of 3. 6 animals per group.
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Figure 6. IL-10 overexpression results in splenomegaly. At day 30 post-IL-10 induction, the spleens of pMT-10 mice present a significative two-fold increase when compared with non-induced mice. The increase of spleen weight is also significative between time points (day 7 and 30), for the induced pMT-10. The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; *** represent a p value ≤ 0.001; One-way ANOVA test. SEM, standard error of the mean. Data are from one representative experiment of 3. 6 animals per group.

Figure 7. Percentage of hematopoietic mature cells over time in the bone marrow or spleen of control and induced pMT-10 mice. The percentage of CD11b^+Ly6C^-Ly6G^- and CD11b^+Ly6C^-Ly6G^- was determined by flow cytometry at days 0, 7, 15 and 30, after zinc-induced IL-10 overexpression. Both in BM (left panel) and spleen (right panel) is observed an increase of these populations over time. The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; *** represent a p value ≤ 0.001; ** represent p value ≤ 0.01; * represent p value ≤ 0.05. One-way ANOVA test. SEM, standard error of the mean. Data are from one representative experiment of 3. 6 animals per group.
Another important feature observed in pMT-10 mouse over-expressing IL-10 is the histological disorganization of the spleen, in which the white and red pulps become undefined (A. Cardoso, unpublished data). Surprisingly, this phenotype is already evident at day 7 (Figure 8), which might indicate that the cellular population orchestrating the splenic disorganization probably belongs to the LSK compartment. Within the spleen, under physiological conditions, HSCs locate close to endothelial cells in the splenic red pulp. Nonetheless, in induced pMT-10 mice the precise localization of the HSCs pool becomes of difficult interpretation. A study from Kiel et al. showed that CD150+ HSCs mobilized to the spleen, during EH, are in contact with sinusoidal endothelial cells in the red pulp (119); nevertheless, it is still undermined whether these HSCs maintain this localization or change it overtime.

Altogether, our results highlight to a defective hematopoiesis, caused by an IL-10-induced stress on the BM, which later leads to extramedullary hematopoiesis along with splenomegaly and abnormal myeloproliferation.

**Figure 8. Spleen histology of control and induced pMT-10 mice.** Figures portray the disorganization of the splenic structure, with a scattered white pulp, in zinc-induced pMT-10 mice, both at day 7 and day 30 after IL-10 overexpression. Control non-induced pMT-10 mice preserve the original architecture of the spleen. Magnification x 4. Represented are the spleens of one mouse per group, from a total of 12, in two independent experiments.
Several animal models show alterations that shift hematopoiesis towards myelopoiesis, progressively leading to splenomegaly. Notably, these models result from abnormal proliferative signals (such as the V617F JAK2 transgenic model) (76), apoptotic cascades (such as the Bim deficient animal) (120), deletion of specific genes with known contribution during hematopoiesis (as the deletion of AML1 gene causes a preleukemia state) (121), or the manipulation of genes involved in cell cycle (such as the cohesin knockdown) (78). In a way, the association of proteins linked with these cellular processes and the establishment of neoplasms is understandable. However, taking into consideration the main role of IL-10 as an anti-inflammatory cytokine, it is remarkable that an overexpression of IL-10 would also lead to hematologic neoplasms. The next section investigates the molecular mechanisms involved in this process.
4.1.2. Molecular Mechanisms Underlying IL-10-driven Myelopoiesis: Inhibitors for JAK2/STAT and PI3K Ameliorate the IL-10-induced Phenotype

The pMT-10 mouse model for induced IL-10 overexpression undoubtedly reflects a phenotype for deregulated BM hematopoiesis, with a shift to myelopoiesis. To determine the molecular pathways involved remains an outstanding question. Among the signaling cascades triggered upon activation of the IL-10R are the JAK-STAT and PI3K-AKT-GSK3 pathways (99, 102). Of these, the most broadly studied is the JAK-STAT cascade, implicated in the majority of the anti-inflammatory responses promoted by IL-10. Recent reports have however revealed the contribution of PI3K in regulating IL-10 functions. Notably, the activation of IL-10R promotes cell survival, by activating PI3K-AKT pathway, which in turn increases the activity of anti-apoptotic molecules.

To assess the involvement of these pathways in orchestrating IL-10-induced myeloproliferation, pMT-10 mice were induced to over-express IL-10 for 30 days after which they were treated with the JAK2/1 inhibitor ruxolitinib (Rx), the PI3K inhibitor buparlisib (Bp) or both for 7 days. The concentration of the drugs used was defined by manufacturer guidelines, for animal’s welfare and reduced toxicity. After the 7 days of treatment, we evaluated the phenotype of pMT-10 over-expressing IL-10 at the BM and spleen levels. In what regards the spleen, both inhibitors led to a significant reduction on its size, thus proving Rx and Bp efficacy in reverting the splenomegaly (Figure 9A and 9B). Furthermore, the histological disorganization, characteristic of the IL-10-induced overexpression (Figure 8), was reverted (Figure 9C), with the spleen of treated animals exhibiting perceptible white and red pulps.
Figure 9. Administration of JAK2 and PI3K inhibitors rescued the splenomegaly induced by IL-10 and ameliorated the histological disorganization of the spleen. Oral administration of JAK2 and PI3K inhibitors for 7 days, after 30 days of induced IL-10 overexpression, recovered the splenomegaly as evidenced by the spleen size (A) and weight (B). The structural organization of the spleen is also regained (C). (A,B) The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; ** represent p value ≤ 0.01. One-way ANOVA test. SEM, standard error of the mean. Data are from 1 representative experiment of 2. (C) Magnification x 4. Represented are the spleens of one mouse per group, from a total of 12, in two independent experiments.
The progenitor and mature cellular populations were analyzed in the spleen (Figure 10) and bone marrow (Figure 11), to investigate if the blockade of the JAK2 and/or PI3K pathways would rescue the physiologic levels of these cells. In the spleen, the percentage of LSKs was only altered in the presence of Bp, showing an appreciable decrease (Figure 10A). However, no significant differences were found for the percentages of GMP or CMP populations (Figures 10B and 10C). As for the mature cells a significant reduction was only observed for Ly6C\(^+\)Ly6G\(^+\) in animals treated with Bp or with both inhibitors (Figure 10E). Thus, the reduction of the splenomegaly observed upon Bp treatment (Figures 8A and 8B) is likely due to the effect of the inhibitor on LSKs and on mature Ly6C\(^+\)Ly6G\(^+\) cells. However, a reduction of splenomegaly upon treatment with Rx is observed (Figures 9A and 9B), despite less significant when compared with Bp. This finding is not associated with a decrease in the cellular populations analyzed (Figure 10); hereinafter, this experiment needs to be repeated or other populations investigated.

![Figure 10. Effect of the administration of JAK2 and PI3K inhibitors on progenitor and mature cells of the spleen.](image-url)

The cellular composition of the spleen was determined for the different experimental groups by flow cytometry. The PI3K inhibitor Bp displays the most significant results, with decrease of LSKs and Ly6C\(^+\)Ly6G\(^+\) when compared to induced pMT-10 mice. Rx administrations appears to not affect the induced-pMT-10 phenotype. The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; *** represent a p value ≤ 0.001; * represent p value ≤ 0.05. One-way ANOVA test. SEM, standard error of the mean. Data are from 1 representative experiment of 2. 6 animals per group.
In what concerns the bone marrow, we observed a significant decrease in the LSK population across all treatments (Figure 11A), although the CMP and GMP populations remained unaltered (Figures 11B and 11C). As for the mature cells, we observed a reversion on the % of Ly6C\(^+\)Ly6G\(^-\), but not of Ly6C\(^+\)Ly6G\(^+\) (Figure 11D and 11E) in contrast to what was found in the spleen. It is possible that a prolonged treatment would result in a better outcome, and perhaps a fully recovery of the hematopoietic cells of the BM, that would lead to cease extramedullary hematopoiesis.

Figure 11. Effect of the administration of JAK2 and PI3K inhibitors on progenitor and mature cells of the BM. The cellular composition of the bone marrow was determined for the different experimental groups by flow cytometry. The PI3K inhibitor Bp, or a combination of both inhibitors, display better results, leading to a statistically significant decrease of LSKs and Ly6C\(^-\)Ly6G\(^-\) populations. The JAK2 inhibitor Rx leads to a reduction of LSKs and Ly6C\(^-\)Ly6G\(^-\), but with less impact when compared to Bp. The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; *** represent a p value ≤ 0.001; ** represent p value ≤ 0.01. One-way ANOVA test. SEM, standard error of the mean. Data are from 1 representative experiment of 2. 6 animals per group.
Therefore, the phenotype induced by IL-10 overexpression was not fully reverted by the tested inhibitors, with the overall best result reported for Bp. Importantly, deregulation of the PI3K signaling is associated with the development of one third of cancers, including lymphocytic leukemia (122). Interestingly, blocking both pathways at the same time did not potentiate the overall action of each single inhibitor. Presumably, other signaling cascades or kinases might be implicated. Both inhibitors seem to act in the BM and spleen, being the population with the most striking differences the LSKs. It is possible that a more prolonged treatment would result in a better outcome, and perhaps a fully recovery of the hematopoietic cells of the BM.

Several studies on cancer signaling reported a synergistic link between JAK and PI3K pathways (123, 124). With our results we can also hypothesize whether, due to the connection between JAK and PI3K, by blocking JAK the most affected signaling cascade is in fact PI3K and not STAT. This theory would explain why solely Rx is not much effective, nor a combination of the two inhibitors increases responsiveness of the cells.
4.1.3. PI3K Signaling Cascade: a Key Process Driving IL-10 Induced Myeloproliferation

Since the previous data supported a greater effect for Bp in rescuing the IL-10-induced phenotype, we next treated pMT-10 mice with Bp for 7 days, starting at the same time of the zinc-induced IL-10 overexpression. The aim was to investigate whether PI3K inhibition could prevent IL-10-induced myeloproliferation.

As shown in Figure 12, blockade of the PI3K pathway attenuated the effect of IL-10, as observed by the absence of splenomegaly and spleen weight maintenance.

![Figure 12. Early inhibition of the PI3K signaling cascade blocks the IL-10 induced phenotype in the spleen. Spleen size (A) and weight (B) after 7 days of induced IL-10 overexpression, in the absence or presence of PI3K inhibitor Bp. Treated mice show an effective blockade of the splenomegaly, when compared to control and induced pMT-10 mice. The data are presented as the mean ± SEM; *** represent a p value ≤ 0.001; ** represent p value ≤ 0.01. One-way ANOVA test. SEM, standard error of the mean. Data are from 1 representative experiment of 2. 6 animals per group.](image-url)
The analysis of the spleen cellular composition upon treatment of pMT10 mice with Bp showed that all the tested populations, from LSKs to mature cells, maintain their normal frequency (Figure 13). The effectiveness of Bp is also evident in progenitors and mature cells of the BM (Figure 14). A decrease on the expanded LSK population is apparent, but not statistically significant (Figure 14A). However, all other tested populations remain within normal percentages, by comparison with the non-induced pMT-10 (Figure 14). Of note, GMPs were also rescued, but still show differences as compared to the control (Figure 14C).

**Figure 13. Administration of PI3K inhibitor Bp blocks the IL-10-induced myeloproliferation in the spleen.** Spleens from the different experimental groups were harvested on day 7 of the experiment and the cellular composition determined by flow cytometry. The data are presented as the mean ± SEM; **** represent a p value ≤ 0.0001; ** represent p value ≤ 0.01; * represent p value ≤ 0.05. One-way ANOVA test. SEM, standard error of the mean. Data are from 1 representative experiment of 2–6 animals per group.
In summary, we showed that blockade of JAK2 or PI3K cascades reverted to a certain extent the IL-10-induced aberrant myeloproliferation. Moreover, inhibition of the PI3K signaling also impaired the phenotype establishment, thus highlighting a potential application of Bp in very early stages of disease. A role for IL-10R induced PI3K-AKT in myeloid cell fate has been proposed before, by triggering a molecular program for inhibition of apoptotic death of myeloid progenitors (125). Therefore, by inhibiting PI3K, the anti-apoptotic role of IL-10 may be blocked. Thus, it is possible that IL-10 may be acting on LSKs and GMPs, by inducing anti-apoptotic programs, leading on the other hand to an exhaustion of CMPs. Also, Flt3 internal tandem
duplications (Flt3-ITD) were found in AML patients, where the constitutive activation of AKT, via PI3K, mediated survival, proliferation and leukemic transformation of myeloid cells, *in vitro* and *in vivo* (126). Thus, PI3K is gaining importance as a promoter of myeloid neoplasms, which supports our data for IL-10-induced myeloproliferation. These hypotheses remain to be investigated and are subject of current studies in our lab.

Importantly, the potential involvement of JAK2 signaling pathway on the phenotype induced by IL-10 should not be disregarded. Inhibition with Rx appears to moderately affect the IL-10 overexpression phenotype. A study by Mascarenhas et al. explored the role of both JAK/STAT and PI3K pathways in HSCs, during embryonic development (127). According to the authors, the JAK pathway has a differential proliferative role, when associated with cytokine-mediated expansion, since it is required for IL-3 signaling, but not for thrombopoietin signaling. Moreover, PI3K inhibition led to a diminished production of hematopoietic progenitors, both in the presence and absence of additional cytokines; this effect was associated with an increase in pre-apoptotic cells (127). These results on signaling pathways of embryonic HSCs support our results on adult HSCs.
4.1.4. Clinical Implications and Outstanding Questions

Unpublished data from our laboratory exposed an unexpected role for IL-10 in deregulating hematopoiesis. Given the relevance of IL-10 in the immune response and the fact that many immune cells produce and respond to IL-10 during infection, as well as the therapeutic potential of manipulating IL-10 in immune related disorders, fully clarifying the role of this cytokine in hematopoiesis is of major importance. The work developed during this thesis aimed at revealing the molecular pathways underlying this novel role for IL-10.

The overall phenotype of the induced pMT-10 mice mimics the phenotype observed in other mouse models for MPN. MPNs are a heterogeneous group of diseases that is characterized by an increase in non-lymphoid hematopoietic cells in the spleen or BM; the classical MPNs are polycythemia vera, essential thrombocythemia and myelofibrosis (61). The JAK2V617F mutation – where JAK2 is constitutively active – was found to be present in many patients diagnosed with MPNs; the discovery of this JAK2 mutation led to the development of several therapeutic JAK2 inhibitors (67). Indeed, Rx is used in the clinic, demonstrating the ability to alleviate some symptoms of the patients, such as to reduce the splenomegaly (68). Nonetheless, administration of Rx does not represent a cure, as the hematological alterations persist in the BM of the patients. These same results were observed when induced pMT-10 mice were treated with Rx.

Due to the heterogeneity of MPNs and the crosstalk between JAK-STAT and other signaling pathways, other non-JAK2 inhibitors have been used, for instance, histone deacetylase inhibitors, and the anti-fibrotic agent pentraxin (61). An interesting study by Choong and colleagues reported the administration of combined inhibitors to treat MPNs in a mouse model for JAK2V617F mutation (123). The authors screened several serine threonine kinase inhibitors, concluding that a more efficient treatment was achieved when combining JAK2 inhibitor – Rx in particular – with a PI3K inhibitor. It was argued that a better therapeutic approach should be accomplished by administering both drugs but at lower doses, since they act synergistically. We observed that PI3K inhibition both reverted and prevented the deregulated myelopoiesis induced by IL-10. Most notably, this effect occurred at the BM and spleen level. Although PI3K inhibitors have proven to be effective in multiple solid tumor types, little is described on its effect on MPNs (128). Additionally, in line
with previous studies (123, 124), we also show a better outcome when administering Rx and Bp rather than Rx alone. Thus, our data highlights the potential of these drugs in MPNs, calling for deeper research in this area, specifically, by evaluating the role of downstream molecules, since the PI3K pathway itself is associated with a plethora of cell functions: metabolism, growth, autophagy, survival. It will also be of interest to assess the effectiveness of PI3K inhibitors in other pre-clinical models of MPNs.

Figure 15 summarizes the main conclusions drawn from the administration of the inhibitors.

![Figure 15. Schematic representation of the pathways involved during IL-10 driven myeloproliferation. Based on our results, both JAK-STAT and PI3K pathways are involved in IL-10-induced myeloproliferation. Administration of JAK2 and PI3K pathway inhibitors Rx and Bp attenuates the myeloproliferative phenotype, after 30 days of induced IL-10 overexpression. However, inhibition of the PI3K cascade might have a more relevant role, as administration of the PI3K inhibitor Bp, at early stages of the establishment of the disease, blocked the progression of the phenotype into a myeloproliferation.](image-url)
4.2. Long-term Effect of IL-10 Overexpression

4.2.1. Sustained IL-10 Overexpression Leads to Premature Death

The induction of IL-10 overexpression on pMT-10 mice triggers numerous cellular alterations that affect the hematopoietic process, inducing a pronounced myeloproliferation. Following these observations, we sustained IL-10 over-expression overtime and found that it culminated with the premature death of the mice. Figure 16 represents the survival curve of induced and control pMT-10. Of note, BL6 animals fed with zinc for the entire period of the experiment (up to day 150) did not die prematurely. Hence the zinc dosage is not toxic. Moreover, induced pMT-10 presented signs of lordokyphosis and lethargy.

![Image of survival curve]

Figure 16. IL-10 overexpression leads to premature death. Mice were maintained in a normal diet or fed with zinc-enriched water and monitored over-time. Control pMT-10 and BL6 animals fed or not with zinc presented a normal survival. pMT-10 mice fed with zinc showed signs of poor health condition and were humanely sacrificed at different times from day 50 onwards. Data are from 1 representative experiment of 2. 6 animals per group.
4.2.2. Sustained IL-10 Overexpression Leads to Blood Alterations Compatible with CMML

To determine the possible cause of death of the IL-10 over-expressing animals, several parameters were measured at an experimental endpoint determined by an evaluation of the animals’ health condition.

Firstly, we hypothesized that prolonged IL-10 overexpression may be driving the development of AML, as the progression of MPNs to AML in human patients is among the most common causes of death (129). To address this hypothesis, blood smears from IL-10 over-expressing animals and controls were obtained and stained with Wright-Giemsa (Figure 17). The first-line diagnostic procedure when a patient is suspected of AML is to obtain BM aspirates or blood to analyze cell morphology. As a routine procedure, blood and BM smears are stained with Wright-Giemsa, to check for blasts; for an AML diagnosis a count of 20% or more blasts is required (130). According to our observations, the blasts found were not enough to assuredly identify the phenotype as AML.
Next, hemograms of pMT-10 over-expressing or not IL-10 were obtained, as hemograms allow a more rigorous diagnosis of certain blood disorders. Figure 18 represents the comparative counts for red blood cells, white blood cells, platelets, the hematocrit, hemoglobin, mean cell volume (MCV), and leukocytes count. As observed, induced pMT-10 mice presented significantly lower values of red blood cells (18A), platelets (18E) and hemoglobin (18C) when compared with controls. Moreover, the induced pMT-10 mice showed values close to the borderline reference ones, indicating that the mice were anemic. This was further supported by the significant decrease on the hematocrit percentage. The anemia was additionally confirmed with Perls staining of liver sections, as it is visible the deposition of iron (Figure 19). The MCV of the induced pMT-10 was elevated when compared to that of control mice (Figure 18D). An assessment of the MCV can elucidate about the typology of anemia; in this context, the increased MCV might imply an aplastic anemia, hemolytic anemia or a myelodysplastic syndrome. Another interesting result
is the decrease in platelets count (Figure 18E), also known as thrombocytopenia. This result is also compatible with a myelodysplastic syndrome or with the development of leukemia. The number of neutrophils and eosinophils appeared to be higher in induced pMT-10 than in the control (Figures 18H and I), whereas the number of basophils did not change upon IL-10 overexpression (Figure 18J). Since AML is defined by neutropenia, these results exclude a typical AML as possible cause of death of the induced pMT-10.
Figure 18. IL-10 overexpression for 60 days induced significant hemogram alterations. The hemograms of control and induced pMT-10 mice were obtained 60 days after zinc administration. Graphics of the counts for red blood cells (A), hematocrit percentage (B), hemoglobin (C), mean cell volume (D), platelets (E), white blood cells (F), monocytes (G), neutrophils (H), eosinophils (I) and basophils (J). The grey area represents the physiological values, also depicted below the graphics as reference values. The data are presented as the mean ± SEM; *** represent p value ≤ 0.001; ** represent p value ≤ 0.01; * represent p value ≤ 0.05. One-way ANOVA test. SEM, standard error of the mean. Data are from 1 representative experiment of 2.
A possible alternative is the development of chronic myelomonocytic leukemia (CMML) in response to IL-10. This hypothesis is compatible with the accumulation of monocytes, increase proliferation of the granulocytic lineage and the evidence of myelodysplasia observed upon overexpression of IL-10 in pMT-10. Indeed, flow cytometry analysis of the spleen and BM of induced pMT-10 (Figures 5 and 7) and the blood analysis (Figure 18) show an expansion of the granulocytic cell lineage (131). According to WHO classifications, the main clinical difference between CMML and other MPNs is the defective hematopoiesis that leads to more persistent anemias and thrombocytopenia, both phenomena also observed in induced pMT-10 mice (60). Furthermore, patients with CMML also display blasts on BM and blood smears, usually below 20%, which is in line with the observations of the blood of pMT-10 + Zinc (Figure 17).

CMML is a rare disease that affects mostly people older than 60 year, being age a risk factor (132). In human patients, the prognosis of CMML is poor, since there is no curative treatment, only therapeutic measures in order to ameliorate the quality of life of the patients, such as drugs to control the myeloproliferation, to repair the anemia and delay disease progression (133). Thus, further investigating the pMT-10 mouse model as a possible CMML animal model may be of interest,
specially considering the previously presented results on the potential of PI3K inhibitors in this setting. It will be of interest to in future test whether PI3K inhibitors may prevent death of the animals over-expressing IL-10.
4.2.3. Prolonged IL-10 Expression Caused Pronounced Alterations in the Skin Histology

To systematically characterize the impact of IL-10 overexpression in pMT-10 mice at the time of death, the histological analysis of various organs was performed (Figure 20 and 21). Firstly, we analyzed the spleen architecture. In line with previous data from our lab, the spleen lacked its characteristic organization (Figure 20). Then, given the fundamental role of IL-10 in gut homeostasis and our own findings showing that the gut is a major IL-10 producer in the induced pMT-10 model (A. Cardoso, unpublished data), we analyzed the histology of this organ. As observed in Figure 20,

Figure 20. IL-10 overexpression leads to splenic histological disorganization, do not affect the gut histology and drives marked changes in the skin histology. The spleen, gut and skin of mice control (left panel) or IL-10 overexpressing (right panel) mice were analyzed by H&E staining 60 days post zinc induction. Splenic disorganization, with loss of white and red pulp areas, is visible upon IL-10-induced overexpression. No obvious histological abnormality was found in the gut of control versus induced pMT-10 mice. Skin histology displayed pronounced alterations in mice over-expressing IL-10. Represented are the histologies from one mouse per group, from a total of 12, in two independent experiments.
the gut did not show any obvious histological abnormality when comparing control and induced pMT-10 mice, thus discarding the involvement of this organ in the sudden death of the animals. In the same line, the histological analysis of the liver and the heart did not display any significant difference between induced and control pMT-10 (data not shown).

The most striking phenotypic difference when comparing the histology from control and induced pMT-10 was found on the skin (Figure 21 and 22). The histologic analysis of the skin allows the distinction between its three main layers: epidermis, dermis and hypodermis. The outer portion – epidermis – is formed mainly by keratinocytes (around 90%); the epidermis layer also comprises the pilosebaceous unit, consisting of hair, hair follicle, sebaceous gland, and arrector pili muscle. As shown in Figure 21, the hair follicle is severely compromised on induced pMT-10. Aged dermis tends to become atrophic (134), with a decrease in fibroblasts and subdermal adipose tissue (Figure 21). Another characteristic difference among aged individuals is the increase in sebaceous glands (134), despite the decrease in sebum production. Similar alterations are observed in induced pMT-10 mice as can be observed in Figure 22.

Figure 21. Sustained IL-10 overexpression induces marked changes in the skin histology. The skin of control (top panel) or pMT-10 mice over-expressing IL-10 (bottom panel) were analyzed by H&E, when mice over-expressing IL-10 were terminally ill. Changes in the skin architecture include an atrophy of the hair follicles (indicated by arrows), shrinking of the dermis (indicated by white lines) and decrease in subdermal adipose tissue (indicated by black lines). These phenotypic alterations are characteristic of an aged individual. The animals used had the same age. Represented one mouse per group, from a total of 12, in two independent experiments.
In line with the aforementioned skin histological alterations, pMT10 animals over-expressing IL-10 present at some point alterations in their physiognomy, most noticeable alopecia and grey hair, whereas control animals with the same age keep a normal phenotype (Figure 23). Moreover, the induced pMT-10 animals show signs of debilitation, and present a hatch up posture – which is an indication of pain and distress for laboratory animals.

![Figure 22. Sustained IL-10 overexpression induces changes in sebaceous glands. H&E skin histology of control (left panel) and IL-10 over-expressing (right panel) mice show augmented size of the sebaceous glands (indicated by black circles) in zinc-induced pMT-10 mice, a feature associated with aging. Control pMT-10 mice appear to have normal sebaceous glands. The animals used had the same age. Represented are of one mouse per group, from a total of 12, in two independent experiments.](image)

![Figure 23. Induced pMT-10 mice present progeroid characteristics upon sustained IL-10 overexpression. Long period of IL-10 induction in pMT-10 mice induces alterations in the animal physiognomy, being the most evident the loss of fur, debilitated appearance and hatch up posture. The picture on the left represents a non-induced control pMT-10 mice; the picture on the right represents an induced pMT-10 mice. Both were of the same age. Represented one mouse per group, from a total of 12, in two independent experiments.](image)
In all, in the presence of sustained IL-10, the animals present signs of premature aging. These include shorter lifespan, lordokyphosis, alopecia with atrophy of the hair follicle, increased sebaceous glands size, thinner dermis and thinner subcutaneous fat cell layers. Notably, these characteristics are among those described as features of an aged individual (Van Deursen et al.) (135). Importantly, the hematopoietic alterations seen in pMT10 mice overexpressing IL-10 also match the characteristics of an aged hematopoietic system, with a bias for the myeloid lineage (81). Future work will assess the presence of other characteristics possibly induced by IL-10, such as bilateral cataracts, muscle atrophy, diminished ability to repair wounds, and also a characterization of the hair follicle stem cells often associated with age-dependent alterations.
4.2.4. IL-10 as a Promoter of Accelerated Aging

Abide by the previous results, where IL-10 overexpression induced the development of progeroid-like features, we questioned if IL-10 was potentiality accelerating aging. To address this question, we resorted to an in vitro model where cellular senescence can be monitored at the molecular level.

With advancing age, senescent cells accumulate in the healthy, injured, or remodeling tissues; moreover, senescent cells are also found in affected tissues of individuals with age-related diseases. A method commonly used to evaluate senescence is to measure the senesce-associated β-galactosidase (SA β-gal) activity (136). SA- β-gal activity is encoded by the lysosomal β-galactosidase gene. Cells usually express lysosomal β-galactosidase activity at a pH of 4.0, but at a pH of 6.0 only senescent cells display β-gal activity. Moreover, the protein levels for lysosomal β-galactosidase increase during senescence. Based on this, we stimulated in vitro cultures of mouse embryonic fibroblasts (MEFs) from BL6 mice, with 10ng/mL of recombinant IL-10 (rIL-10) (Figure 24). As a control, MEFs not treated with rIL-10 were included. Fourty-eight hours later, cells were fixed and stained to detect β-gal activity. Interestingly, addition of 10ng/mL of rIL-10 programmed MEFs into senescence, with a percentage of around 6% associated with the presence of more than 10 β-gal-containing lysosomes per cell. This is the threshold normally used to undoubtedly consider cells to be senescent.
We next started to investigate the mechanisms and signaling pathways involved in IL-10-induced cellular senescence. A well-characterized senescence-activating molecular cascade is that mediated by the tumor suppressor p53, which leads to the activation of p21 (137). In fact, p21 is a marker for cell cycle arrest, associated with the inhibition of cellular proliferation (137). As such, the lack of DNA replication, due to the inhibition of proliferation, is itself a good marker for senescence and can be measured by ki67 expression. To investigate possible molecular mechanisms for IL-10-induced senescence in MEFS, we stimulated the cell cultures with rIL-10 for 48 hours, as before. At that time point, the cells were fixed and stained with fluorescent antibodies for p21 and ki67, to assess cell cycle arrest and DNA replication, respectively. Results were obtained in the IN Cell Analyzer 2000 and automatically analyzed with the equipment software, by establishing brightness, contrast and intensity thresholds to distinguish positive from negative cells. Preliminary results showed that IL-10-treated MEFs displayed an increase in p21 activation, when compared to non-treated control, indicating that IL-10 is acting
via p21 (Figure 25). As for ki67 detection, no differences were found, independently on the dose of rIL-10 used (Figure 24A). Having in consideration that our aforementioned results suggest that IL-10 signals through PI3K, and being PI3K a pathway essential to cell survival and proliferation, it is possible the existence of a conflicted signaling pattern, on one hand activating senesce through p21 and on the other hand sustaining cell survival.

The fact that TGFβ is reported to trigger senescence in a paracrine manner (138), and IL-22 – a cytokine of the family of IL-10 – being evidenced to promote senescence in HSCs (139), fuels the hypothesis that IL-10 is a player in organism aging. Further studies will be performed to further investigate the molecular pathways activated by IL-10 and leading to cellular senescence. These include p16, due to its relevance in senescence and since it is independent of p53 cascade (137), and the autophagic profile of the cells. Given the importance of PI3K pathway in our work, we will monitor the activation of the PI3K cascade in MEFs in the presence or absence of IL-10 and test its functional relevance by chemically inhibiting it.

Figure 25. Recombinant IL-10 alters the p21 signaling cascade in mouse fibroblasts. The percentage of ki67 and p21 positive cells in MEF cultures was determined by immunofluorescence upon stimulation with 0, 10 or 50ng/mL of rIL-10 for 48h. Data are presented as the mean ± SEM; *** represent p value ≤ 0.001; One-way ANOVA test. SEM, standard error of the mean. Represented is one experiment performed.
4.2.5. Placing IL-10 as a Modulator of Hematopoietic Senescence: Future Perspectives

Aging, defined as a time-dependent loss of physiological functional integrity, is a natural process that affects every system and every organism. A better understanding of the mechanisms driven by aging is crucial to assure a better lifestyle (140). Of importance, the process of hematopoietic immunesenescence should be extensively studied, since a deteriorated hematopoiesis has life-threatening consequences (80). Our animal model for IL-10 overexpression has features that resemble the phenotypic traits of an aged hematopoiesis, characterized by impaired lymphopoiesis and increased myelopoiesis. Those reports drove us to hypothesize a possible role for IL-10 in aging. With that purpose, we established a systematic study for prolonged IL-10 overexpression, and an in vitro study to determine the extent of IL-10 action. Altogether, our results established that IL-10 is able to induce premature aging in the pMT-10 model, and senescence in fibroblasts cell cultures targeting the activation of p21 – a marker for senescence and cell cycle arrest.

Upcoming work needs to performed in order to thoroughly characterize the induced pMT-10 model, by examining other progeroid features such as the development of bilateral cataracts and the diminished ability to repair wounds. Given the intrinsic association between aging and thymus involution, our group is also interested in investigating the process in induced pMT-10 mice, to determine if IL-10 overexpression accelerates the phenotype. Moreover, focusing on the skin, it is of relevance to assess the activation of p21 also in MEFS of pMT-10 mice, by administering zinc throughout the pregnancy and establishing a MEF primary cell culture of the progeny. Other signaling cascades may play a role, particularly p16 – a canonical mediator of senescence independent of the p21 pathway (137). Furthermore, it is of interest to investigate the activation of autophagic programs.

The significance of the PI3K signaling cascade in the pMT-10 mouse model was determined by the administration of a PI3K inhibitor in the animals overexpressing IL-10 (chapter 4.1 of the thesis). Moreover, other reports show that p53-p21 signaling pathway can be activated through PI3K (141). This suggests that PI3K might be involved in the IL-10 induced senescence; therefore it is crucial to evaluate PI3K activation also in MEF cell cultures, allowing a corroboration with the in vivo model.
Finally, once established a clear role for IL-10 in inducing senescence, our group aims to try to revert it at molecular level; thus revealing the alterations imposed by IL-10 to the progression of the cell cycle, during the process of immunesenescence. Figure 26 presents a schematic illustration of the purposed model for IL-10 in promoting premature aging.

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**Figure 26.** Which signaling cascades are implicated in IL-10 induced senescence? Preliminary work in MEF cultures suggests that IL-10 induces cell senescence via p21. Given the relevance of PI3K signaling, we hypothesize that response to IL-10 is made via PI3K, leading to subsequent activation of p21. Hereinafter, we will also study a possible role for p16 in IL-10-induced senescence.
5. Final Conclusion

IL-10 is an important anti-inflammatory cytokine now evidenced to affect hematopoiesis by shifting cell differentiation towards myelopoiesis and likely involved in cellular senescence. It is tempting to speculate that these findings are actually related, and as such, in addition to the future plans to further address each of these findings, we also plan to pursue them in an integrated way.

Hematopoiesis is a highly regulated process, which accompanies all changes of the organism (7). Moreover, any inadequacy during cell differentiation programs can lead to an overall deregulation, culminating in life-threatening pathologies. Findings from the current thesis place the pMT-10 mouse of IL-10 overexpression as a possible model for CMML, due to its hematological features. Of interest, CMML is a disease more prone to be developed in older individuals (132), in line with the other age-related features found in this model. The striking hematologic results, the shorter life-span and skin histology strongly raise the possibility of the induced pMT-10 showing a progeroid syndrome. This may be linked to IL-10-driven senescence, since senescent cells drive age-related pathologies. Preliminary results conducted *in vitro* suggest that in fact IL-10 induced senesce via p53-p21 signaling cascades. These observations offer IL-10 a broader importance within organism homeostasis, now placing it as a modulator of (anti-) inflammmaging and immunosenescence. Importantly, there are precedents in the literature linking anti-inflammatory cytokines with senescence, as studies on mesonephros and endolymphatic sac, during development, found that p21 upregulation, and consequent cell senescence, are controlled by TGFβ-SMAD and PI3K-forkhead box protein O pathways (142). Moreover, another member of the IL-10 family, IL-22, has also been described to promote senescence in HSCs (139). Thus, our findings and others in the literature fuel the hypothesis that IL-10 may be a player in organism aging.

Immunosenescence is described as the process that the hematopoietic system undergoes with aging, which leads to increased susceptibility to infectious diseases, anemias, autoimmune diseases, and even cancers (80, 143). Among these alterations are the diminished number of naïve T cells; increased number of myeloid precursors (which sometimes originates neoplasms); decreased functional capacity of mature myeloid cells; and increased number of HSCs, but with loss of regenerative capacity (81). Understanding the mechanisms driving
immunosenescence, associated with aging, is of great interest considering the current demographic shift towards aged individuals, and also the association of many diseases – of neoplastic or neurodegenerative nature – with advanced aging. Given the role of IL-10 in myelopoiesis and as a promoter of senescence that we now present, studying IL-10 in the context of immunosenescence might guide future therapeutic strategies for aged-associated hematologic diseases.
6. References


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7. Annexes

The poster presented at SPI – Sociedade Portuguesa de Imunologia – annual meeting XLII 2016 entitled “Immunity, From Cells to Organisms” follows as an annex. The poster reflects the work developed during the second part of my thesis (chapter 4.2).
Exploring the role of IL-10 in aging

Guilherme M. Cardoso1,2,*, Ana Cardoso1,2,*, Joana Calafate Macedo1,2, Isabel Castro4,5, Ana Cumano2, Elsa Loganin7,8, Antonino G. Castro4, Paulo Vieira4,6 and Margarida Sarafkina1,2,*

1-2-3-4-5-6, University of Porto, Portugal
2-7, Immune Regulators Group, BMC, University of Porto, Porto, Portugal
3-5, Aging and Immunity Group, BMC, University of Porto, Porto, Portugal
4-5, Life and Health Sciences Research Institute (ICVLS), School of Health Sciences, University of Madeira, and ICVLS/STI: Government Associate Laboratory, Braga/Guimarães, Portugal
6-7, Unit Lymphocytes, Instut Pasteur, Paris, France

Introduction

Aging is defined as a time-dependent loss of physiological functional integrity. The cellular deterioration associated with aging is often the foundation of many pathologies, such as cancer, cardiovascular, metabolic and neurological diseases. The aging process is also evident in the immune system, reflecting the production of immune cells, the ability to respond to infections and increasing the incidence of certain hematological disorders. In particular, immune senescence is characterized by impaired lymphopoiesis and increased myelopoiesis.

Interleukin (IL-10) is an anti-inflammatory cytokine critical for homeostasis during immune responses. Through the study of a mouse model of inducible IL-10 over-expression, the pMT-10 mice model, we have recently uncovered a role for this cytokine as a regulator of hematopoiesis. Over-expression of IL-10 leads to pronounced myelopoiesis, with an increase in granulocytic/myeloid progenitor cells, accompanied by anemia, thrombocytopenia and reduced B lymphopoiesis.

Changes induced by IL-10 in hematopoiesis resemble those observed in aged hematopoietic systems. Moreover, mice over-expressing IL-10 die prematurely and present other programmed features of accelerated aging, including shortened stature, very thin skin and senility. Since the role of IL-10 in aging, the extent of the studies and the inherent molecular pathways are largely unknown, we are currently addressing these issues. For this purpose, we are studying in a systematic way the histological and functional alterations imposed by IL-10 over-expression on different organs, from the blood and bone marrow to the liver and the skin.

Aims

Our data place IL-10 as a possible modulator of hematopoietic senescence. The aims of the current work are:

- To investigate why sustained IL-10 expression culminates in the premature death of the animals.
- To explore the alterations promoted by sustained IL-10 over-expression in the hematopoiesis of different organs.
- To understand the molecular determinants of IL-10-mediated senescence.

Future Perspectives

- What signaling cascades orchestrate aging downstream to the L. LIF?
- Can we reverse it?
- What alterations does IL-10 impose on the progression of the cell cycle?
- How extensive is the impact of IL-10 on the aging process?
- What are the functional consequences of the IL-10-induced alterations?

Results

Sustained IL-10 over-expression leads to premature death of pMT-10 mice

pMT-10 mice over-expressing IL-10 terminally develop chronic myelomonocytic leukemia

IL-10 over-expression leads to pronounced histological changes in the skin

pMT-10 mice present profound alterations upon sustained IL-10 over-expression. Long period of IL-10 injection in pMT-10 induces alterations in the animals, in the extent of the liver and spleen, in the shape of the red and white blood cell, in the structural integrity of the organs, in the shape of the myocardial cells, in the density of cortical vessels induced pMT-10 mice. Of note, the gut is a major IL-10 producer in the induced pMT-10 model (not shown).

**In IL-10 accelerating aging?**