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Over-expression of IL-10 by PMT-10 mice: a novel model of myeloproliferative diseases?

Ana Catarina Matos Cardoso Silva

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia, realizada sob a orientação científica da Professora Doutora Margarida Saraiva (Escola de Ciências da Saúde da Universidade do Minho) e do Professor Doutor Paulo Santos (Faculdade de Ciências e Tecnologia da Universidade de Coimbra)

Ana Catarina Matos Cardoso Silva

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ABBREVIATIONS

ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
BM	Bone Marrow
BudR	Bromodeoxyuridine
CFU	Colony forming units
CLP	Common lymphoid progenitors
CLR	C-type lectin receptors
CML	Chronic myelogenous leukemia
CMP	Common myeloid progenitors
DC	Dendritic cells
EBF	Early B cell factor
ET	Essential thrombocythemia
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-Macrophage colony stimulating factor
GMP	Granulocytic-Macrophage Progenitors
HSC	Hematopoietic stem cells
HE	Hematoxylin-Eosin
IFN	Interferon
IL	Interleukine
IL-10R	IL-10 Receptor
JAK	Janus Kinase

MegE	Megakaryocytic-Erythroid Progenitors
MPD	Myeloproliferative disorders
MDS	Myelodyplastic syndrome
MPN	Myeloproliferative neoplasm
MYD88	Myeloid differentiation primary response gene (88)
NK	Natural killer
PB	Peripheral Blood
PMF	Primary Myelofibrosis
PV	Polycethemia Vera
RBC	Red blood cells
RT-PCR	Reverse transcriptase- polymerase chain reaction
STAT	Signal transducers and activators of transcription
SyK	Spleen tyrosine kinase
Th	T helper
TLR	Toll like receptors
TRIF	TIR-domain-containing adapter-inducing interferon-β
WHO	World health organization
Zn	Zinc

KEY WORDS

- Hematopoiesis
- Hematopoietic Stem Cells
- Interleukin-10
- Common myeloid progenitors
- Myeloproliferative Disorders

ABSTRACT

Several cytokines, such as IL-3, IL-6 and IL-12, involved in inflammatory immune responses, have been described as relevant on the modulation of hematopoiesis. However, the role of anti-inflammatory cytokines, like IL-10, has not yet been addressed. We used genetically modified mice that over-express IL-10 under the control of a zinc inducible promoter, PMT-10 mice, to address this question.

We show that over-expression of IL-10 led to a splenomegaly, with increase of total number of cell numbers and the appearance of megakarycocytes. Interestingly, we observed a substantial increase in myeloid cells and a decrease in B-lineage cells both in the spleen and the bone marrow (BM) upon IL-10 over-expression. Furthermore 30 days after induction of IL-10 expression, a severe reduction in lymphoid precursors and an expansion of myeloid precursors was observed in the BM. Collectively, our data suggest that IL-10 over-expression impacts hematopoiesis, inhibiting the differentiation of B cells and stimulating the differentiation of the myeloid lineage.

Moreover, the phenotype observed after IL-10 over-expression is similar to that seen in human patients and in mouse models of myeloproliferative disorders (MPD), namely polycythemia vera (PV), thrombocythemia (ET) and primary myelofibrosis (PMF).

MPD are frequently caused by mutations on the JAK2 gene, which are present in 90% of patients with PV and 50% of the patients suffering from ET or PMF (11). Based on the epidemiology of MPD and on the phenotype observed for IL-10 over-expressing mice, we hypothesize that increased IL-10 may associate with susceptibility to MPD, explaining the 10 - 50% of cases unrelated to the JAK2V617F mutation. Therefore, PMT-10 mice are of potential interest as a novel model to study the molecular mechanisms leading to MPD, in the context of non-mutated JAK2. In future we will explore these possibilities, as well as the mechanism by which IL-10 interferes with hematopoiesis.

RESUMO

As citoquinas envolvidas na resposta imune inflamatória, tal como a Interleucina (IL)-3, IL-6 e IL-12, possuem um papel de grande relevância na modulação da hematopoiese. Contudo o papel de citoquinas anti-inflamatórias, como a IL-10, não foi ainda esclarecido. Usando ratinhos geneticamente modificados que sobre expressam a IL-10 sob o controlo de um promotor activado por zinco – murganhos PMT10 – obtivemos evidências que sugerem que essa sobre expressão de IL-10 tem um impacto a nível da hematopoiese, inibindo a diferenciação das células B e estimulando a diferenciação da linhagem mielóide.

Os nossos dados preliminares indicam que os ratinhos PMT10 sujeitos ao tratamento de zinco apresentam uma esplenomegalia quando comparados com os grupos controlo, sendo esse aumento acompanhado de uma desorganização estrutural do baço. Conjuntamente, o número total de células no baço aumenta, alterando a proporção normal das diferentes populações celulares aí residentes. Apesar de o número total de células na medula permanecer inalterado, tanto a percentagem como o número de células das diferentes populações é alterado.

Este perfil é semelhante ao perfil observado nas Doenças Mieloproliferativas, nomeadamente, policitemia vera (PV), trombocitemia (ET) e mielofibrose primária (PMF). Uma mutação adquirida no gene JAK2 – $JAK2^{V617F}$ – é encontrada em 90% dos casos de PV, e em 50% dos pacientes que sofrem de ET ou PMF. Está descrito na literatura que a IL-10 sinaliza via JAK/STAT. Com base nisso, propomos a IL-10 como um bom candidato para explicar os outro 10-50% de casos não relacionados com a mutação $JAK2^{V617F}$ e, os ratinhos PMT10 como um potencial novo modelo de estudo de doenças mieloproliferativas.

1. INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis is a continuous and regulated process that ensures differentiation of hematopoietic stem cells (HSCs) into mature blood cells, maintaining homeostasis and guaranteeing protection against pathogens [1-3]. Blood cells have a short life, being constantly renewed through proliferation of immature cells in the hematopoietic organs. Differentiation of HSCs occurs in fetal liver, spleen, liver, thymus, lymph nodes and bone marrow (BM) but, throughout adulthood it occurs mainly in the BM [1, 3, 4].

The HSCs have the ability to repopulate the hematopoietic tissues as well as of maintaining themselves but, when they undergo differentiation, they give rise to a progeny that progressively loses it self-renewal capacity and becomes restricted to one lineage [1-3, 5]. It is believed that the decision of self-renewal versus differentiation, with loss of potential for differentiation into other cell fates [1-4, 6-11], is random whereas the later differentiation would be determined by signals delivered by the stromal microenvironment [2]. The identification of stem and progenitor cells by Weissman and collaborators [6, 10, 12-15] led to the construction of a hematopoietic lineage tree that is characterized by a cascade of binary decisions. In the BM investigators identified a common progenitor for all lymphoid lineages, the common lymphoid progenitors (CLP) [10], as well as a common myeloid progenitor (CMP) that generates granulocytic and macrophages (GM) [6]. The observation that CMPs and CLPs derived from adult BM generate mutually exclusive progeny [6, 10] suggests that their diversification represents the earliest branching point during hematopoietic differentiation. CLPs give rise to pro-B and pro-T cells, uncommitted lymphoid progenitors that will differentiate further into mature B and T cells [10]. GM and Megakaryocyte (Meg) lineages give rise to granulocytes, macrophages and natural killer cells (NK), and megakaryocytes and erythrocytes, respectively [6]. The offspring of GM also includes neutrophils, eosinophils, and possibly basophils/mast cells [16, 17].

Unlike all other lineages, which are specified in the BM, T cells differentiate after migration of early progenitors into the thymus [18]. Interestingly, was also found that CLPs are able to origin NK lineage cells [10].

Curiously, functionally equivalent and phenotypically indistinguishable myeloid and lymphoid dendritic cells (DCs) can be derived from either CMPs or CLPs [19, 20]. A schematic representation of hematopoiesis is in figure 1.

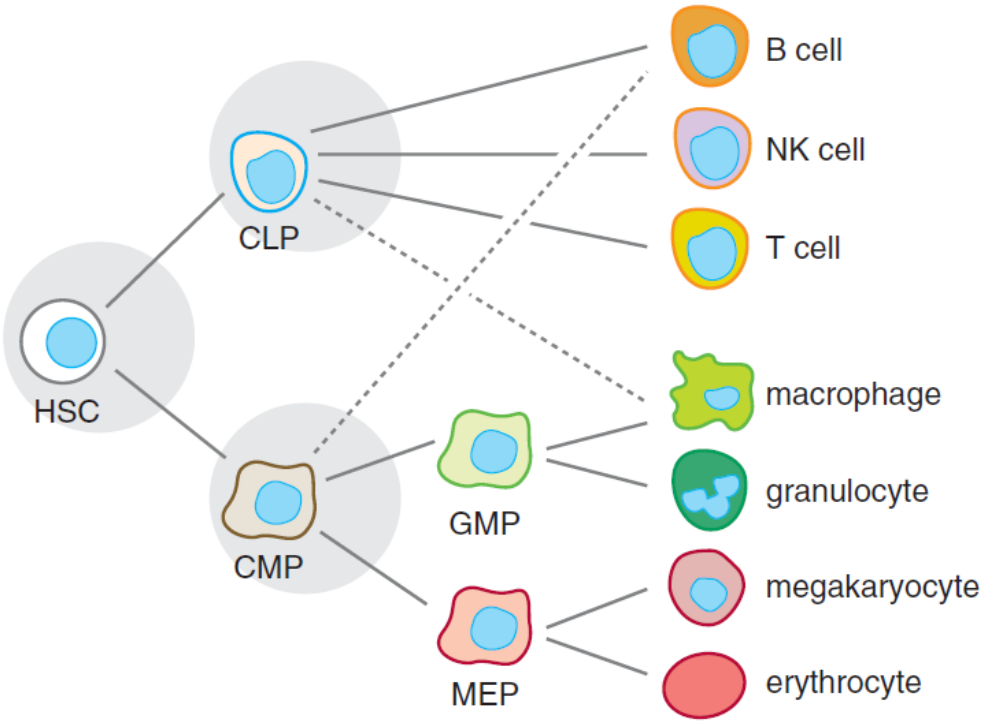


Figure 1. Representative scheme of the hematopoietic process.

The establishment of all hematopoietic lineages during development is tightly controlled by transcription factors that act in sequential and parallel fashions, building lineage-specific networks or circuits [7, 21-23]. Lineage commitment can be induced either by extracellular factors, including cytokines, direct cell-cell interactions, or other environmental cues [5, 9, 24]. Alternatively, intrinsic mechanisms, such as the stochastic upregulation of transcription factors, or other regulatory molecules, such as microRNAs can also induce lineage commitment [25, 26]. Both extrinsic and intrinsic factors may either have an instructive role and actively induce commitment and differentiation or be merely permissive for the outgrowth of pre-committed progenitors by promoting cell survival and/or expansion [24].

The role of specific transcription factors in regulating hematopoiesis has been addressed in much detail. B cell development requires a complex set of transcription factors, namely PU.1, Ikaros, early B cell factor (EBF), E2A, and Pax5, and inactivation of any of these factors yields a severe phenotype [27-31]. The dramatic impairment of B cell development in PU.1-deficient mice has been ascribed to lack of expression of the PU.1 target genes IL-7Rα and EBF [28, 30] because B cell differentiation is impaired in mice deficient for either of these genes [29, 31]. Loss of EBF blocks B cell development at the pro-B cell stage before initiation of B cell receptor (BCR) rearrangements [29]. A similar phenotype was described for E2Adeficient mice [27].

Many macrophage- and granulocyte-restricted promoters are regulated by PU.1 and/or C/EBPα [32]. These factors cooperate in the regulation of the genes encoding the myeloid growth factor receptors MCSFR, G-CSFR, and GM-CSFR [32, 33]. Mice deficient in C/EBPα lack neutrophil and eosinophil granulocytes, and conditional inactivation in the BM shows the specific absence of GMPs and reduced numbers of CMPs, leading to decreased formation of all downstream lineages [34, 35]. In GATA-1-deficient embryos, development of erythroid cells is blocked early in differentiation, leading to a lethal anemia [36, 37].

Furthermore, several evidences suggest that not only transcription factors have regulatory roles in hematopoiesis, but also cytokine receptor signalling. The involvement of cytokines in hematopoiesis will be addressed more deeply in the next chapter.

1.2 Cytokines and Hematopoiesis

The work of many groups over the past years has uncovered a great deal of information regarding the mechanism by which cytokines modulate hematopoiesis [5]. Numerous transcription factors are induced by cytokines in the microenvironment where lymphopoiesis and myelopoiesis are occurring to control the expression of cell–specific genes. Early observations show that IL-3 is a positive regulator of early erythropoiesis causing rapid cell proliferation and increased cell survival of myeloid progenitors [38-41], acting also synergistically with several other cytokines, such as IL-6 [42, 43] and G-CSF on committed and uncommitted myeloid progenitors [44]. It has been shown that IL-7 plays an essential role in B cell differentiation, due to the fact this cytokine is uniquely able to maintain the expression of EBF in BM derived CLP and cause the expansion and survival of committed B cell progenitors (pro-B cell) [45]. Lymphopoiesis is accompanied by a severely reduced expression of EBF in CLP [46]. Several studies demonstrated that the age-related impairment in BM IL-7 impacts also T lymphopoiesis, since in the presence of mutations in IL-7 or IL-7R gene result in a dramatic block of T cell development in the thymus [45, 47-50].

Moreover, it has been established that IL-7 promotes proliferation, survival, and development of pro-B cells towards the pre-B cell development [51, 52]. Interestingly, a previous study shows that the administration of IL-7 stimulates the proliferation of the myeloid lineage indirectly by the action of IL-3 and G-CSF [53]. Moreover, it has been reported that IL-3 expands an early cell population in vivo that subsequently requires the action of a later acting factor such as GM-CSF to complete its development [54]. GM-CSF also presents an important role in cell differentiation since GM-CSF deficiency leads to impaired pulmonary homeostasis and increased splenic hematopoietic progenitors, but unimpaired steady-state hematopoiesis [55]. Like IL-7, IL-21 has also been shown to play a key role in B cell development [56]. However, in contrast to IL-7, IL-21 exerts its effects not only at early stages of the development, but also at later stages, inducing the growth and differentiation of mature B cells into Ig-secreting plasma cells [57, 58]. IL-6 is able to support emergency granulopoiesis in animals that lack G-CSF and GM-CSF, two critical cytokines

necessary for myelopoiesis [59, 60]. Interestingly, has been shown that IL-6 blocks lymphopoiesis elevating the production of myeloid cells in lupus erythematosus [61]. Recent studies show that IL-12 plays a significant role in vivo hematopoiesis by suppressing hematopoiesis in the BM, enhancing peripheral (splenic) hematopoiesis and mobilizing hematopoietic progenitor cells to the peripheral circulation [62, 63]. Other cytokines, such as Type I IFNs, are known to strongly inhibit proliferation of pro-B cells and consequently B cell development [64]. IFNα in particular, is able to activate dormant HSCs (in vivo), by promoting their efficient exit of G0 phase and entrance in the active cell cycle, thus stimulating the proliferation of HSCs. However, IFNαR mediated signalling is not required for the HSCs function [65].

In all the role of cytokines, mainly pro-inflammatory ones, in hematopoiesis has been addressed and their contribution to the hematopoietic decision there is well established. A schematic representation of this network in figure 2.

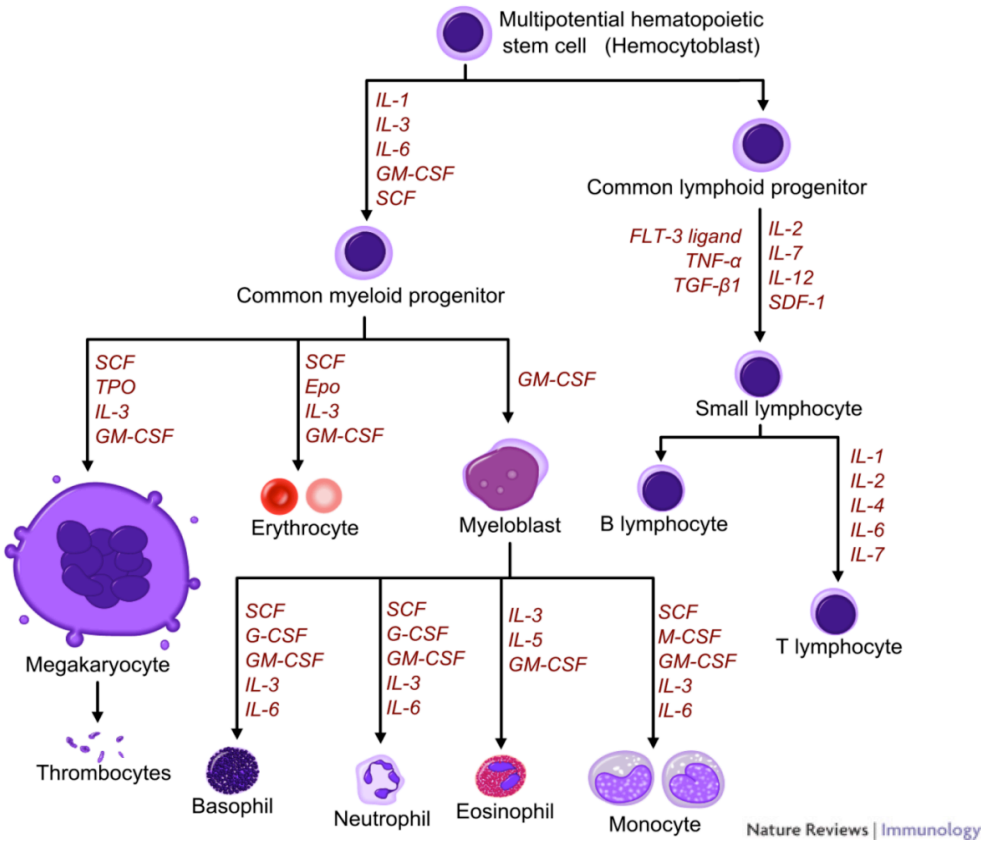


Figure 2. Schematic representation of the hematopoiesis regulatory molecules.

1.3 IL-10: an anti-inflammatory cytokine

IL-10 is an anti-inflammatory cytokine produced by many types of immune cells, from both lymphoid and myeloid lineages, presenting a central role in infection [66]. T cells are an important source of IL-10, since many subsets of T cells can produce IL-10, such as Th2 cells, natural regulatory T cells, originated in the thymus and inducible regulatory T cells, originated in the periphery [67-69]. Recently, Th1 and Th17 subsets were also found to produce IL-10 [70-72], together with IFNγ or IL-17, respectively, when induced by particularly strong antigen dose and inflammatory responses, allowing the immune responses to be inherently self-regulating [67]. Moreover, IL-10 is produced by monocytes, macrophages and DCs upon activation of toll like receptors (TLRs) [73, 74] and other pattern recognition receptors (PPRs) [75].

Most hematopoietic cells express the IL-10 receptor (IL-10R) whereby IL-10 is able to regulate several different steps of the innate and adaptive immunity [66]. The IL-10R comprises two subunits, IL-10R1 that is expressed in most hematopoietic cells and IL-10R2, which presents a crucial role in the recruitment of the signalling molecules Janus Kinase (JAK) [66, 76, 77]. Stimulation of IL-10R-expressing cells with IL-10 leads to activation of JAK1 and Tyk2, and STATs 1 and 3 [78]. STAT3 is recruited directly to sequences surrounding two membrane-distal tyrosines in the IL-10R [79]. A schematic representation of IL-10 signalling is in figure 3.

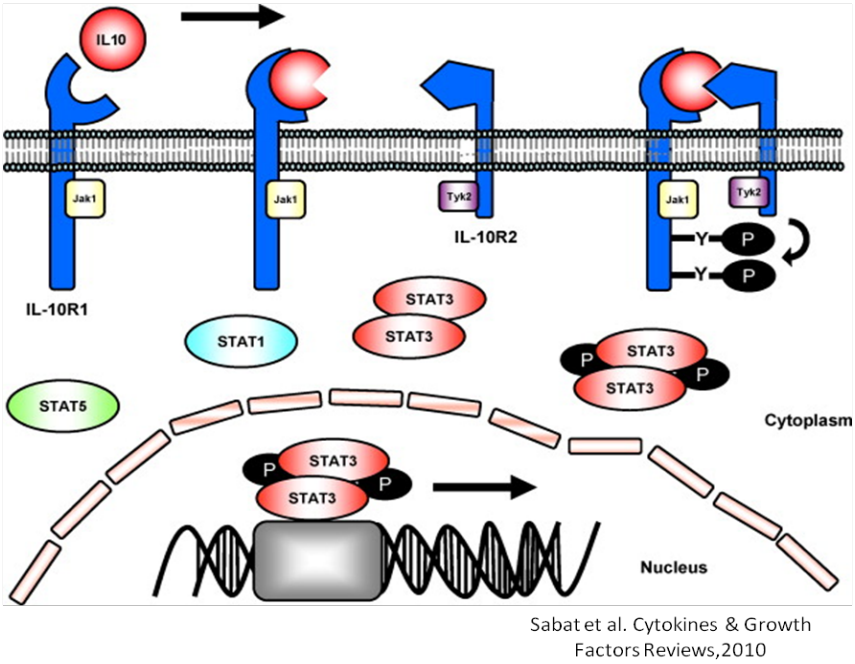


Figure 3. Schematic representation of IL-10 signalling pathways.

IL-10 can inhibit the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 and TNF, as well as the expression of chemokines implicated in the recruitment of monocytes, neutrophils, T cells and DCs [80]. In addition IL-10 can affect T cells directly, limiting their capacity to proliferate and produce cytokines, such as IL-2 and IFNγ [81, 82], or promote the differentiation of naïve T cells into IL-10-producing regulatory T cells [68]. In contrast, IL-10 also have stimulatory effects on mast cells [83, 84] and B cells by upregulating the expression of MHC class II molecules and enhancing IgA responses [85]. IL-10 can induce the recruitment, proliferation and cytotoxic activity of NK cells and CD8 T cells [86]. A schematic representation of IL-10 effects is in figure 4.

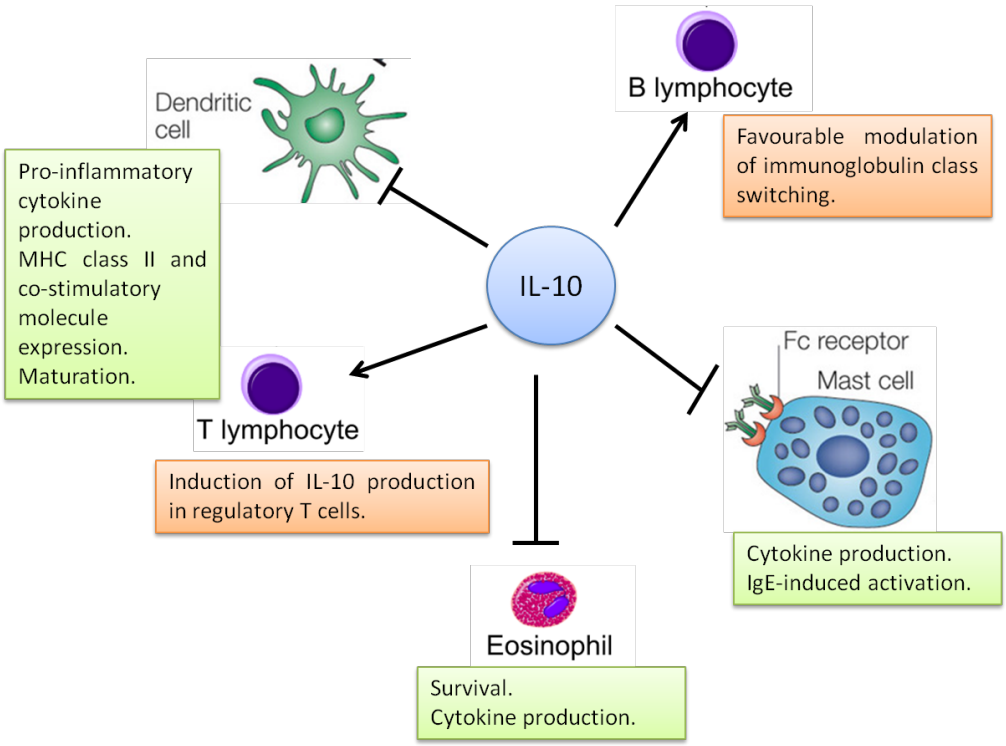


Figure 4. Representative scheme of IL-10 effects.

1.3.1 IL10 in infection

The immune system comprises all structures and processes that provide defence against potential pathogens. These defences can be divided into two different responses, the innate immunity and the adaptive immunity [87, 88]. The key difference between innate and adaptive immune response is the promptitude of innate immune response that uses a pre-existent repertory but limited of response components. In contrast, the adaptive immunity response albeit slow in activation, present the capacity of recognizing a large repertory of strange substances and the ability of increasing the effectiveness of the response while innate immunity remains constant [87, 88]. As discussed above IL-10 is produced during infection by cells of the innate and acquired system, in support of an important role throughout the immune response. In myeloid cells

IL-10 is induced following TLR ligation in response to an overabundance of pathogen products. The magnitude of IL-10 induction within different myeloid cell types has been associated to the relative strength of extracellular related kinases 1 and 2 (ERK1/2) activation [89]. The induction of IL-10 in myeloid cells in response to TLR-ligands requires the signalling adaptor molecules MYD88, TRIF and numerous indirect pathways mediated by autocrine/paracrine factors [69, 90-92]. In addition to IL-10 induction by TLR-dependent stimuli, IL-10 can also be induced from innate cells such as DC via TLR-independent stimuli including C-type lectin receptors (CLR), via the spleen tyrosine kinase (Syk) dependent pathway [93].

1.3.2 IL-10 in disease

IL-10 has an important role in the pathogenesis of numerous disorders presenting contradictory effects. IL-10 over-expression is associated with several autoimmune disorders, such as Lupus erythematosus and certain lymphomas, through the action of its immunosuppressive effects [94-97]. Moreover, IL-10 deficiency is connected with rheumatoid arthritis, psoriasis, Crohn's disease and untreated patients suffering from multiple slecorosis [98-101]. Interestingly, associations between IL-10 allelic variations and the risk of development of these disorders have been established. For example the IL-10 polymorphisms, T-3575A, G-2849A and C-2763, that lead to an increase of IL-10 expression, are associated with an increasing risk observed in patients with Lupus erythematosus [102, 103]. Finally, IL-10 also regulates the immune responses to pathogens. In this context, excess of IL-10 is often associated with chronicity, whereas its absence can cause collateral damage to the host due to an excessive uncontrolled pro-inflammatory response [104].

The pleiotropic effects of IL-10, demonstrated by many [94-100, 105-109] has led to a variety of clinical studies to employ recombinant IL-10 or anti-IL-10R in the treatment of patients with immune mediated diseases [110].

1.3.3 IL-10 in Hematopoiesis

As mentioned before, most hematopoietic cells express IL-10R, thus are potentially targets of IL-10. However, the role of IL-10 in hematopoiesis has not been fully addressed. However, there are some evidences, gathered over the years, suggesting that IL-10 may influence the hematopoietic process. A possible role for IL-10 in hematopoiesis was firstly illustrated, through its growth-promoting activity on mast cell, synergistically with IL-3 and IL-4, megakaryocytes and multilineage colonies derived from committed progenitors and $Thy1^{low}$ $SCA1^{+}$ [84]. Furthermore, Vlasselaer et al showed, in vitro, that IL-10 was able to stimulate the production of granulocyte/macrophage colony forming unit by blocking the TGF-β synthesis by the osteogenic stroma, promoting hematopoiesis in this microenvironment [111, 112]. There are also evidences indicating that IL-10 acts synergistically with erythropoietin significantly increasing erythroid differentiation and proliferation in vitro [113]. Interestingly, a bidirectional effect of IL-10 on early B cell development has been reported, by stimulating Flt3 ligand with IL-7, IL-10 can induce pro-B cell formation and growth before differentiation into a more mature form (CD19 expression). On the other hand, IL-10 has been suggested to inhibit the growth of pro-B cells after differentiation, expressing CD19 [109]. An inhibitory effect of IL-10 on GM-GSF in vitro was reported, suggesting that this cytokine may have potential in the treatment of myeloid malignancies that are linked with mechanisms underlying GM-GSF deregulation [108]. As detailed in section 2 of this thesis, our main goal was to further investigate the role of IL-10 in hematopoiesis.

1.4 Deregulation of the hematopoietic process

As mentioned before, normal hematopoiesis is a highly regulated process that consists on a strict balance between self-renewal and differentiation of HSCs. Numbers and fate decisions made by progenitors derived from HSCs must be carefully regulated to sustain large-scale production of blood cells. As mentioned before, Interleukins, IFNs and other molecules are of great importance in regulating these processes, delivering critical cues to HSCs and progenitors. Several of these molecules have been connected with malignancies and are causatively involved in the development of several types of hematopoietic disorders such as leukemias, lymphopenias or myeloproliferative disorders (MPD) [51, 58, 95-97].

Leukemias are a group of heterogeneous neoplastic disorders of white blood cells. Based on their origin, myeloid or lymphoid, they can be divided into two types. Leukemias traditionally have been designated as acute or chronic, based on their untreated course [114]. Acute lymphocytic leukemia (ALL) is a malignant clonal disorder of the BM lymphopoietic precursor cells [114]. In T cell ALL, progressive medullary and extramedullary accumulations of lymphoblasts that lack the potential for differentiation and maturation are present. The involvement of IL-7 in T cell ALL through the modulation of cell cycle regulators such as cyclins D2 and A has been shown [115]. In acute myeloid leukaemia (AML), the BM produces too many early blood cells, that aberrantly express Nuclear factor κB, which do not go on to become mature blood cells [114-116]. Excessive production of immature myeloid cells ultimately prevents the normal production of RBC, resulting in anaemia, and decreased production of platelets or thrombocytopenia. [116-118]

Due to their possible relation to the data presented in this thesis, MPD are detailed in the next sections.

1.4.1 Myeloproliferative Disorders

MPD embrace several clonal hematologic diseases that are thought to rise from a transformation in a HSC [114, 119-121]. The main clinical feature of these diseases is the overproduction of mature and functional blood cells [114, 119, 120]. MPD occur primarily in the BM, but sometimes in the liver and spleen. According to World Health Organization (WHO) guidelines, myeloid neoplasms with 20% or more blasts in the peripheral blood (PB) or BM is considered to be acute myeloid leukemia (AML). This specific MPD is a result of a molecular lesion in the Philadelphia chromosome BCR-ABL gene, which most commonly results from the Philadelphia translocation [122]. Evolution to AML can also happen, in the context of a previously disorder such as myeloproliferative neoplasm (MPN), myelodyplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN). In AML, as in ALL, a proliferative of blasts in extramedullary site can also occur [114].

The three main MPDs negative for the Philadelphia translocation are Polycethemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) [114, 120]. PV is characterized by the massive production of mature red blood cells (RBC) in the BM, generally accompanied by an increase of megakaryocytes and other members of the myeloid lineage [114, 119-121]. Erythroid precursors in PV are extremely sensitive to erythropoietin, leading to an increase of RBC production [114]. Moreover, precursors in PV are more responsive to cytokines such as IL-3, GM-CSF and steel factor. Megakaryocytes increase also in the BM although more than 60% of patients present endogenous megakaryocytes colony unit formation [114, 120, 123]. As a result of the increase in RBC production, an increase of blood viscosity is normally observed in these patients, leading to thrombosis and/or bleeding [114, 119, 120]. People suffering from PV present a hematocrit between 53% and 62% [114], while 43-48% in healthy individuals, being the hematocrit values proportional to the number of thrombotic events [119, 124]. Increased platelet numbers also contribute to these events. An enlargement of the spleen is also common and occurs around 75% of PV cases [114, 123]. This disorder is very rare in children and young adult, occurring in a very late stage of life, whereby

its prevalence is not very high, surrounding 3-100.000 persons per year in USA [114, 123].

PMF, in similarly to PV, is caused by an expansion of HSCs in the BM [114, 119, 120]. In PMF, cell proliferation is accompanied by a reactive nonclonal fibroblastic proliferation and fibrosis of the BM [114, 119, 120]. The proliferation of HSCs leads to the production of several factors, such as platelet derived growth factor, epidermal growth factor and basic fibroblastic growth factor, causing the fibrosis of the BM [119, 124]. As the BM becomes fibrotic and normal hematopoiesis can no longer happen, extramedullary hematopoiesis occurs in the spleen – leading to a splenomegaly and liver enlargement [121]. As seen in PV, PMF is developed in an advanced stage of the adult life. The prevalence of this disorder in the USA is of 1-100.000 persons per year [123].

ET, the last of the BCR-ABL negative MPD, is characterized by a constant proliferation of megakaryocytes in the BM, with an increased number of PB platelet counts [114, 119]. In this disorder, megakaryocyte progenitors are hypersensitive to the action of several cytokines, such as IL-3 and IL-6 [119, 121]. The increase of platelets leads to a growth of thrombotic and hemorrhagic events [119, 121]. A splenomegaly is also common, affecting proximately 30% of the patients [114, 119, 121]. Although it can occur at any age ET usually affects older people, with most patients diagnosed between ages of 50 and 70 years. Like PV, ET is a rare disease diagnosed in 3-100.000 persons per year in USA [123].

These three disorders are closely related, sharing many characteristics including a hypercellularity of the BM, a propensity to thrombosis and haemorrhage and a risk of leukemic transformation in a clinical long term. The main criteria for identifying MPDs reside in an initial diagnostic of the PB and BM by observing the blast percentage. Blast cells should be derived when possible from 200 cell leukocyte differential counts of the PB smear and 500 cell differential counts of all nucleated BM cells on cellular marrow aspirate smears stained with Wright-Giemsa or similar stain [114]. Moreover, flow cytometry is the method of choice to determine the blast lineage as well as for detecting aberrant antigenic profiles. It is essential to determine a baseline karyotype by performing a complete cytogenic analysis, as well it is to repeat it when the goal

is to judge the response to a therapy or for detecting genetic evolution. Additional genetic studies should be guided by the results of the initial karyotype, and by the diagnosis suspected based on the clinical, morphological, and immunophenotypic studies. In some cases, reverse transcriptase-polymerase chain reaction (RT-PCR) may detect variants of well recognized abnormalities, like JAK2, MPL, KIT and GATA1 in MPDs [114, 125-129].

A certain number of criteria are required for distinguishing the MPDs subtypes not only from each other, but also from reactive granulocytic, erythroid, and/or megakaryocytic hyperplasia that often mimics myeloproferative neoplasms (MPNs). Interestingly, it has been show that in chronic myelogenous leukemia (CML), the massive cell proliferation is caused by rearrangements/mutations of certain genes that encode surface or cytoplasmatic protein tyrosine kinase, that lead a constitutive activation of the signal transduction pathways[114, 120, 125-130]. Some of the genetic abnormalities that responsible for MPDs, such as the mutated JAK2 or KIT, are not specific but provide proof that the proliferation is clonal and thus, when present, eliminate further consideration of a reactive process [114, 131].

Currently, the most commonly recognized mutation in BCR-ABL negative MPDs is JAK2 V617F [120, 129, 130, 132]. This mutation is found in more than 90% of patients with PV and proximately 50% of those with ET or PMF [114, 130, 133]. Furthermore, in PV patients that lack this mutation, a similar activating JAK2 exon 12 mutation can be found [134], and a small proportion of patients with PMF and ET of approximately 10% who lack JAK2 mutation may instead demonstrate activating mutations of c-MPL, such as MPL W515K or MPL W515L, which produces a protein that responds to a growth factor that stimulates platelet production [128, 135, 136].

1.4.2 $JAK2^{V617F}$ Mutation

The JAK family comprises four members, JAK1, JAK2, JAK3 and Tyk2, which share significant structural homology with each other [137, 138]. The kinases associate constitutively with a variety of cytokine and hormone receptors. JAK1, JAK2 and Tyk2 are expressed throughout the body, while

JAK3 is mainly expressed in lymphoid and myeloid cells [137, 138]. Signal transducers and activators of transcription (STAT) proteins are a family of transcription factors that bind as homodimers or heterodimers to recognition sites in gene promoters. STATs are the result of seven different genes, STAT1, STAT2, STAT3, STAT4, STAT5A, STATB and STAT6 [137-139]. STAT proteins are selectively phosphorylated by different families of protein kinases. Some cytokines which receptors lack intrinsic tyrosine activity rely on the JAK family to phosphorylate the STAT proteins [137].

Upon binding of the specific ligands to their receptors, JAK kinases are rapidly activated and their kinase activities induced, to regulate tyrosine phosphorylation of various effectors such as STATs and initiate activation of downstream signalling pathways: when phosphorylated STATs dimerize and become activated, they lead to the transcription of important genes for cell survival, activation and proliferation [137]. The JAK/STAT pathways mediate signalling of several membrane receptors such as Interleukins, IFNs and growth factors receptors, being the most common cause of abnormal JAK activation a deregulated cytokine signalling [139-141].

Three of these kinases, namely JAK1, JAK2 and Tyk2 have been shown to be involved in IL-10 signalling pathway. Finbloom et al observed that IL-10 treatment of T cell and monocytes resulted in a ligand-induced tyrosine phosphorylation of Tyk2 and JAK1 [78]. Furthermore, Gupta et al demonstrated recently that the IL-10/IL-10R complex upregulated JAK2 signalling [78, 142].

A mutation in the JAK2 gene, consistent of a single point alteration in JH2 auto-inhibitory domain, leads to a substitution of the aminoacid valine by phenylalanine at position 617 (V617F), resulting in constitutively activated kinase [125, 143]. V617F mutation, as mentioned before, is found in a large proportion of people suffering from MPDs.

The discovery of this mutation in the JAK2 gene was of great importance since its opened new horizons in diagnosis and treatment of MPDs. In fact, recent studies using transgenic animals expressing the $JAK2^{V617F}$ mutation have brought new insights into the world of MPDs [144, 145], suggesting that the chronicity of human MPN may be a reflection of a balance between impaired HSCs function and the accumulation of additional mutations, but not only from alterations of the HSCs compartment [146-148]. Molecular testing for

similar mutations, but in particularly for the JAK2^{V617F} mutation, plays a key role in the diagnosis of BCR-ACL negative MPDS as a way of separating neoplastic from myeloid proliferations [114].

Activating alleles of JAK2, such as JAK2 ^{V617F}, are essential to the development of MPDs, suggesting that small molecule inhibitors targeting JAK2 may be therapeutically useful. Several studies have reported the development of new drugs targeting JAK2, such as anagrelide, CYT387 and ruxolitinib [149-151]. CYT387 has been shown to normalize the hematocrit values, spleen size, white cell counts and the physiologic levels of inflammatory cytokines [151] as well as ruxolitinib [150]. Interestingly, when in the absence of JAK2 mutations these inhibitors are also efficient in improving MPD [149-151].

1.5 Aims

Understanding the molecular mechanisms that regulate hematopoiesis is of great importance, considering that this process is responsible for the production of all blood cells and that its deregulation leads to fatal diseases. Among the factors regulating hematopoiesis are cytokines, such as IL-3, IL-7 or GM-CSF. However, the role of anti-inflammatory molecules, such as IL-10, in hematopoiesis has been less studied. Taking advantage of our IL-10 over-expressing inducible model, PMT-10, in this thesis we propose to understand the role of IL-10 over-expression in hematopoiesis. For that we will:

- Determine the impact of the IL-10 over-expression in the differentiation of the different cell lineages;

- Study the mechanisms underlying this differentiation;

- Evaluate the plasticity of our mouse model in recovering from the phenotype induced by IL-10 over-expression.

2. MATERIAL AND METHODS

Animals: PMT10 mice on a C57BL/6 background were generated by Drs. António G. Castro and Paulo Vieira. A mouse IL-10 cDNA sequence was cloned into the p169ZT vector, which carries a sheep metallothioneinc (MT) Ia promoter, a β—globin splice site and a polyadenylation signal. The resulting vector – pMT-IL10 – was then injected into C57BL/6 eggs and transgenic founders were identified by PCR using MT and IL-10 specific primers. IL-10 overexpression was induced by giving the mice a 2% sucrose solution with 50mM of zinf sulfate to the animals. Since the IL-10 promoter is associated with a metalloprotein, the presence of zinc (Zn) in the solution induces its activation. A group of transgenic littermates were supplied with regular water as a control. All animals were bred under specific pathogen-free conditions at ICVS.

For all experiments, a group of PMT-10 mice was induced to over-express IL-10 [152] for 30 days. As a control, 3 different groups were used – wild type mice exposed or not to Zn and non-treated PMT-10 mice.

ELISA: The concentration of IL-10 in the mice serum was determined by immunoassay using the mouse ready-set-go mouse IL-10 ELISA kit (eBioscience).

Cell Suspensions: Cell suspensions from the spleen and thymus were prepared by removing the organs and meshing them with a syringe pistol in ice-cold cDMEM (Gibco). BM cell suspensions were prepared by removing the femur and tibia and sectioning its extremities. Using a syringe with a needle containing cDMEM the BM was flushed through the bones. The spleen, thymus and BM suspensions were then gently dispersed through a 40μm pore size nylon tissue strainer (BD Biosciences); the resultant cell suspension from the spleen, BM and blood was treated with Erythrocyte lysis solution to remove residual red blood cells, washed and counted. These cells were used for western blot and flow cytometry analysis.

Histological studies: All excised tissues were fixed in 3,7% formaldehyde and included in paraffin. Additionally, femurs were decalcified using Biodec R from Bio Optica. Sections or the organs (2-4μm) were processed for light microscopic

studies after hematoxylin-eosin (HE) staining and analysed using (Olympus BX61).

Immunoblotting: Protein extracts were prepared from cell suspensions from the spleen and BM by lysis in Lysis Buffer. 40 µg of protein lysates were resolved on 10% sodium-docecyl sufalte - 8% acrylamide gels, and transferred to a membrane (BioRad trans-blot turbo transfer pack) using Trans-Blot Turbo Transfer Starter System. Detection of the total and phosphorylated forms of JAK2 was performed using specific antibodies from Cell Signalling.

Blood Smears: Smears were performed using 5 µl of blood removed from the tail or eye, fix and stained with Wright Stain (SIGMA).

Microhematocrits: Hematocrit values were measured at different time points by collecting of tail or eye blood in heparinised capillary tubes (Hirschman Laborgerate). These were then centrifuged at 12000rpm for 6min and RBC were compared using a hematocrit gauge.

Separation of Lin$^-$ cells: Single cell suspension from the BM were subjected to magnetic separation in an AUTOMACS (Mylteni) using antibodies in biotin for CD3, CD4, CD8, CD11c, CD11b, NK1.1, CD117, TER-119, B220, CD19 (Biolegend, eBioscience and BD Pharmingen). The negative fraction – Lin- fraction – was then analysed through flow cytometry to determine the expression SCA1, c-Kit, FcγR and CD34 (Biolegend, eBioscience and BD Pharmingen).

Flow cytometry analysis: Immunofluorescence staining was performed to determine the expression of CD3, CD4, CD8, CD11b, CD11c, CD19, B220, GR1 and Ly6G ((Biolegend, eBioscience and BD Pharmingen) on cell suspensions from the blood, spleen, BM and thymus. Lin- cell suspension from the BM was stained with specific antibodies for SCA1, c-Kit, FcγR and CD34. In brief, $1x10^6$ cells were incubated for 10 min at 4ºC with Fcblock (Biolegend) in FACs buffer. Cells were then stained with primary antibodies for 30 min at 4ºC.

Cells were analysed using a BD Biosciences FACSCalibur flow cytometer and data analysed using FlowJo 7.6 software.

Statistical analysis: The results are given as means ±SE. Statistical significance was calculated using one way ANOVA. Values of $p \leq 0.05$ were considered significant.

3. RESULTS

3.1 Characterization of PMT-10 mice

PMT-10 mice are transgenic modified animals able to over-express IL-10 under the control of a Zn-inducible sheep-metallothionein protein [152]. With the goal of characterizing the general features of PMT-10 mice under Zn administration, a group of PMT-10 mice was induced to over-express IL-10 for 30 days, the approximate period of time it takes to renewal the hematopoietic cells in the peripheral blood. IL-10 promoter was induced by administering, in the drinking water, a solution of Zn sulphate to PMT-10 mice. As a control, 3 different groups were used – wild type (WT) mice exposed or not to Zn and non-exposed PMT-10 mice (figure 5).

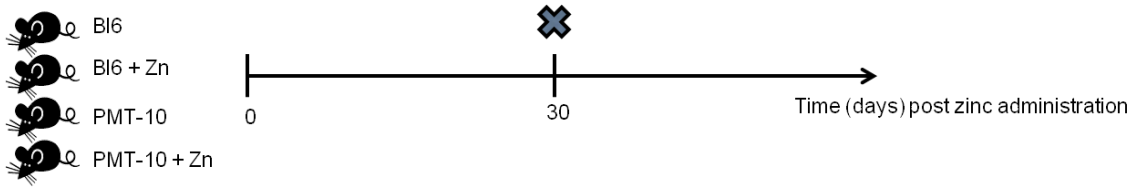


Figure 5. Experimental layout.

3.1.1 IL-10 kinetics in Zn treated PMT-10 mice

In order to confirm that Zn treated PMT-10 mice were over-expressing IL-10 we measured the amounts of seric IL-10 by immunoassay, as shown in figure 6. The levels of IL-10 in WT, WT exposed to Zn and non-treated PMT-10 mice, in the serum of peripheral blood were below the detection level (BDL) of the assay, at all the time points analysed. In Zn treated PMT-10 mice high IL-10 amounts were detected at day 5 (5 days after Zn-mediated induction), increasing until day 15 and held up to day 30, when the IL-10 induction with Zn was ended.

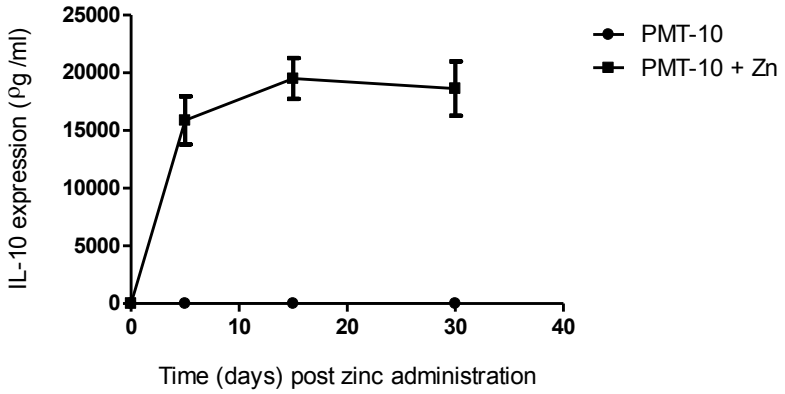


Figure 6. IL-10 kinetics in PMT-10 mice exposed to Zn.PMT-10 mice were treated with Zn for 30 days to induce over-expression of IL-10. At the indicated time points, IL-10 concentration was determined by ELISA in the serum of the induced PMT10-mice and all control groups – WT exposed or not to Zn and non-treated PMT-10 mice. Data from one experiment representative of three from 6 mice per group.

3.1.2 Over-expression of IL-10 induces splenomegaly, accompanied by an increase of total number of splenocytes and spleen weight

After showing that Zn administration for 30 days induced high and sustained IL-10 expression (figure 6) next we analysed the impact of this over-expression on lymphoid organs. For that, spleens were collected and compared between the Zn-exposed PMT-10 mice and the control groups. As show in figure 7a, the induced PMT-10 presented a pronounced splenomegaly. This enlargement of the spleen was accompanied by a sharp increase of the total number of cells, reaching approximately 450 million of cells compared to the100 million cells in WT exposed or not Zn and non-treated PMT-10 mice (figure 7b). The spleen weight in treated PMT-10 mice also increased (figure 7c). All control groups – WT exposed or not to Zn and non-exposed PMT-10 mice, maintained a normal spleen size and weight, with no alterations of the total number of cells (figure 7a-c).

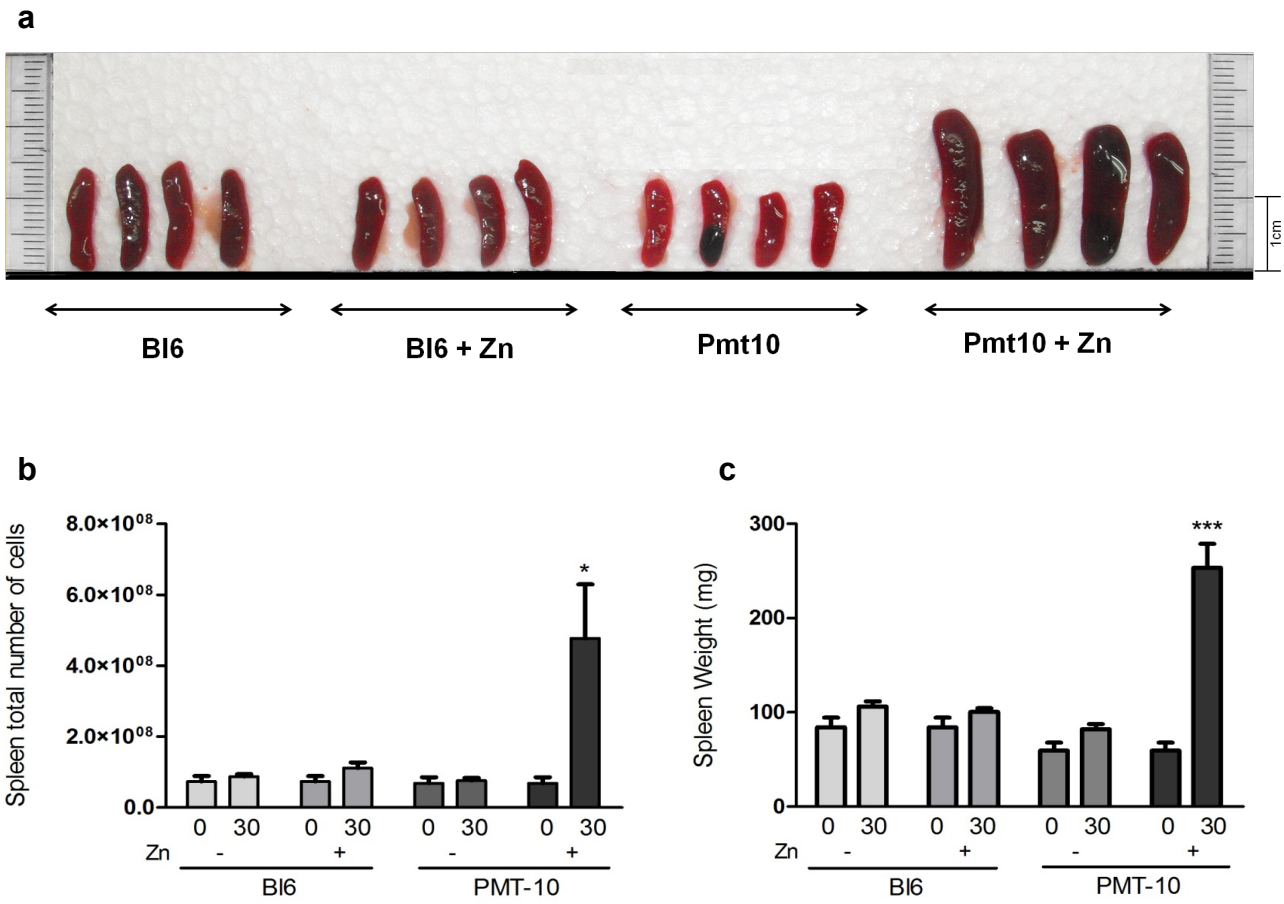


Figure 7. IL-10 over-expression causes splenomegaly in PMT-10 mice. PMT-10 mice were feed for 30 days with Zn to over-express IL-10 at which time spleens were removed and the size (a), total number of cells (b) and weight (c) were measured in control groups. Data from one experiment representative of three, with mean ± SEM from 5-6 mice per group, one way ANOVA * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

3.1.3 IL-10 over-expression leads to the structural disorganization of the spleen

Samples from spleens harvested after 30 days of Zn exposure were processed for histological studies by staining with HE. As show in figure 5a, the spleens from WT mice shows a typical histological profile were its visible both the white and red pulp. The same profile is seen in WT mice with Zn as well as exposed PMT-10 mice. In contrast the spleens of IL-10 over-expressing PMT-10 mice show a tissue structural disorganization (figure 8a). Interestingly, an

increased number of megakaryocytes, a typical feature of extramedullary hematopoiesis can be seen in PMT-10 mice over-expressing IL-10 (figure 8b).

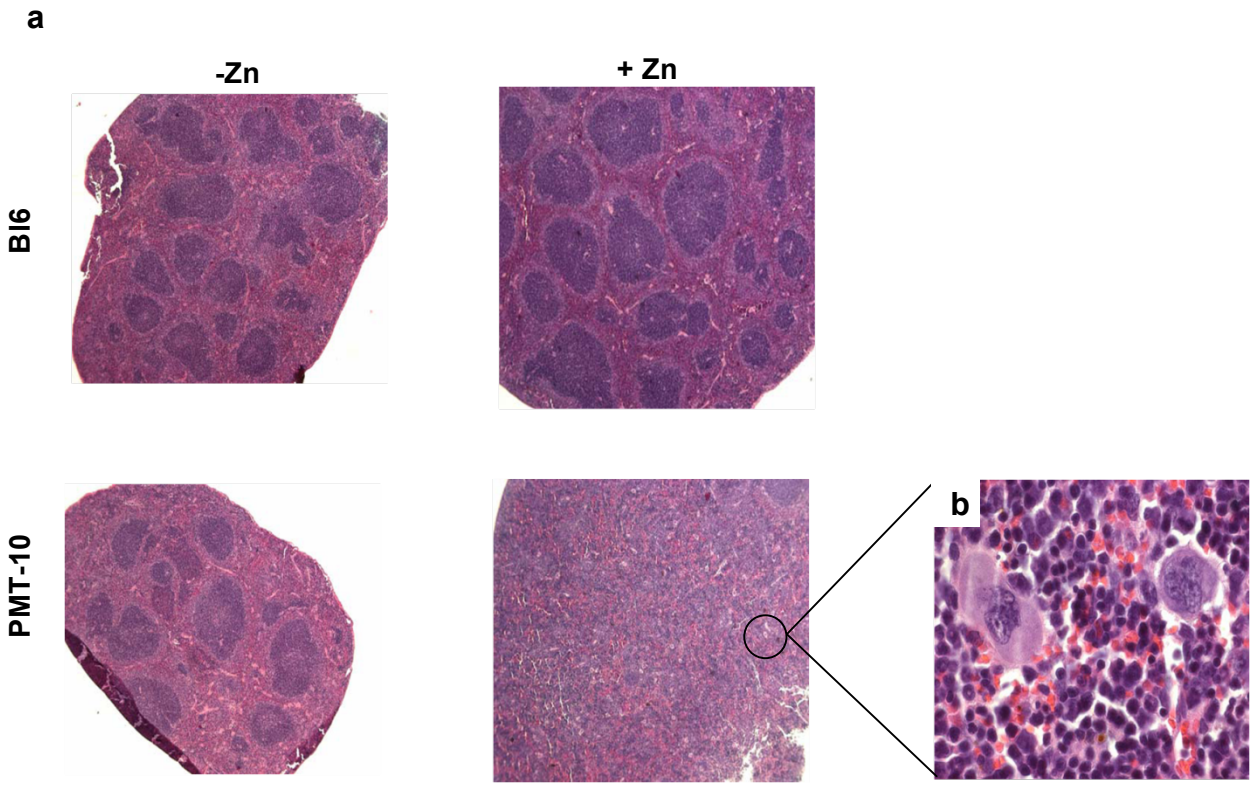


Figure 8. Structural disorganization of spleens of PMT-10 mice over-expressing IL-10. The PMT-10 mice were subjected to Zn for 30 days, inducing the IL-10 over-expression. Spleens from WT and PMT-10 mice exposed or not to Zn were removed at day 30, and samples from each group were histological analysed by HE stain. Each image shows one mouse representative of each group (a). Observation of giant cells morphologically similar to megakaryocytes at 400x (b).

3.1.4 IL-10 over-expression does not affect the thymus

Given the massive alterations caused by IL-10 in the spleen, we next evaluated the possible role of IL-10 over-expression in the thymus due to its involvement in cell differentiation and proliferation. To access this, at day 30 of

IL-10 over-expression in PMT-10 mice, thymus from all groups were removed and analysed for their total number of cells and subsets (CD3, CD4 and CD8) by flow cytometry. As shown in figure 9, none of the groups presented any differences in the total number of cells (figure 9a) or in the different subsets (figure 9b).

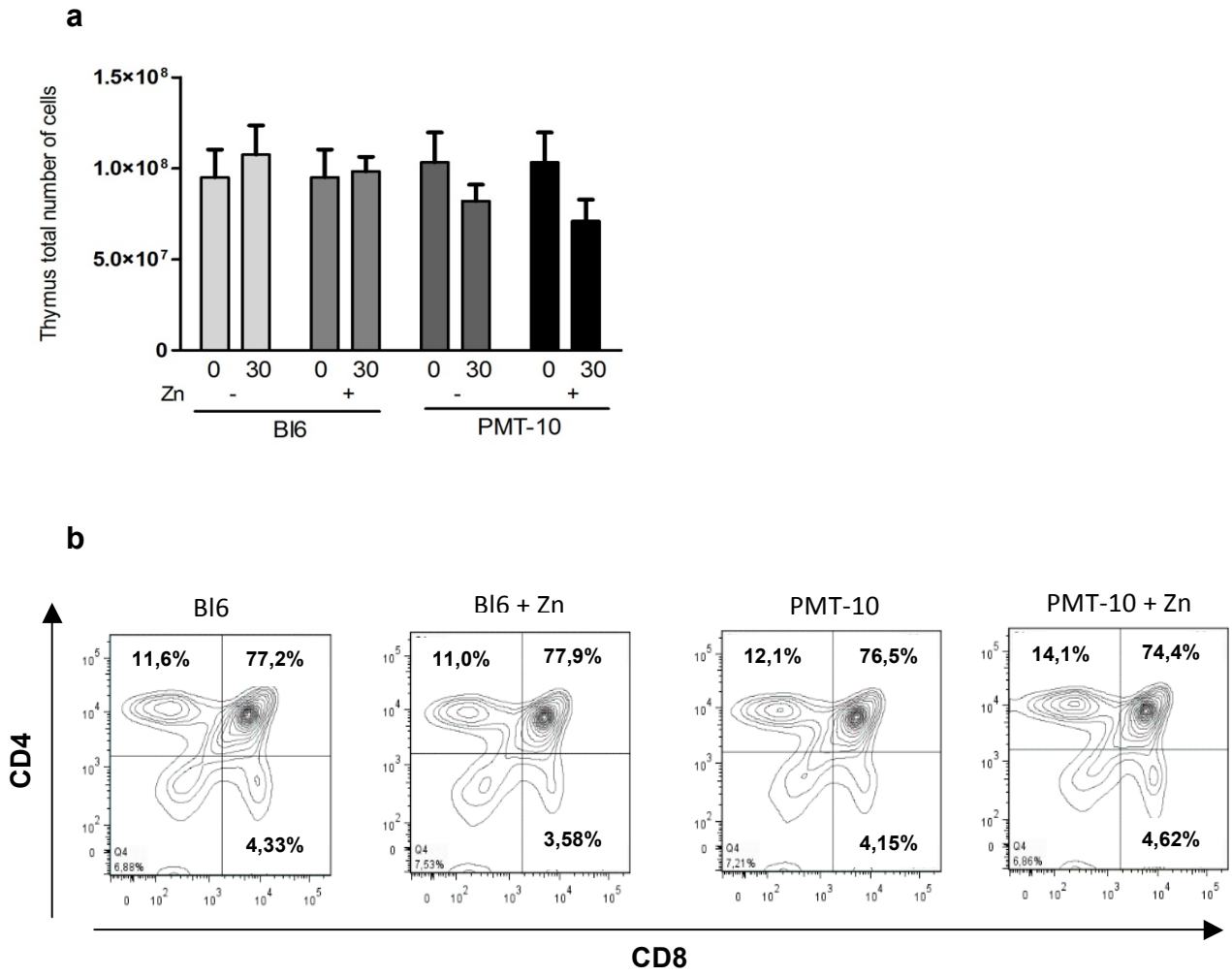


Figure 9. PMT-10 mice over-expressing IL-10 present a normal thymus over time. PMT-10 mice were induced to over-express IL-10 with Zn. At day 30 of IL-10 over-expression, thymus from all groups were harvested and analysed for their total number of cells. The total number of cells was determined using the Cell Counter (Countess™ Invitrogen). Data from one experiment representative of three, with mean ± SEM from 5-6 mice per group, one way ANOVA * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

So far, our results suggest that high levels of IL-10 produced by PMT-10 mice exposed to Zn impacts hematopoiesis. The splenomegaly, increased cell numbers and structural disorganization of spleen, and increased number of megakaryocytes shown in PMT-10 mice and caused by IL-10 over-expression are features seen a specific group of hematopoietic disorders, the MPD. The profile presented by MPD patients and mouse models [114, 119-121], is in line with the characteristics seen in PMT-10 mice over-expressing IL-10, adding a new perspective to IL-10 functions.

3.2 Impact of IL-10 over-expression in hematopoiesis

3.2.1 IL-10 over-expression leads to changes in spleen cell populations

Since we observed a massive increase in the spleens of the PMT-10 mice exposed to high levels of IL-10, we next investigated whether a normal distribution of cellular populations was occurring. To test this, we induced IL-10 over-expression by feeding PMT-10 mice with Zn for 30 days and evaluated the major leukocyte subsets by flow cytometry. In WT mice exposed or not to Zn and non-exposed PMT-10, the total number of B and T lymphocytes and myeloid cells, in the spleen, was maintained over time (figure 10a-f). PMT-10 mice over-expressing IL-10 presented a total number of cells of the B and T cell subsets similar to the other groups (figure 10a and 10c). However, the percentage of these populations significantly decreased (figure 10b and 10d) suggesting the increase of other cellular populations. Indeed, we observed a sustained increase on the number and percentage of the myeloid lineage. These alterations characterized by a decrease of the lymphoid lineage in favour of the myeloid lineage that leads to a sharp increase of total cell number and consequently to a splenomegaly, are similar to the ones seen in MPD patients [114, 119-121]. In these patients, the growth of the myeloid lineage is a consequence of an extramedullary hematopoiesis in the spleen with loss of BM hematopoiesis [114, 119-121]. Seen this we questioned whether, in PMT-10 mice over-expressing IL-10, the observed increase of the myeloid population was specially caused by (i) a proliferation of HSC in the spleen – extramedullary hematopoiesis, (ii) the transference from the BM or (iii) a synergistic effect between the two organs.

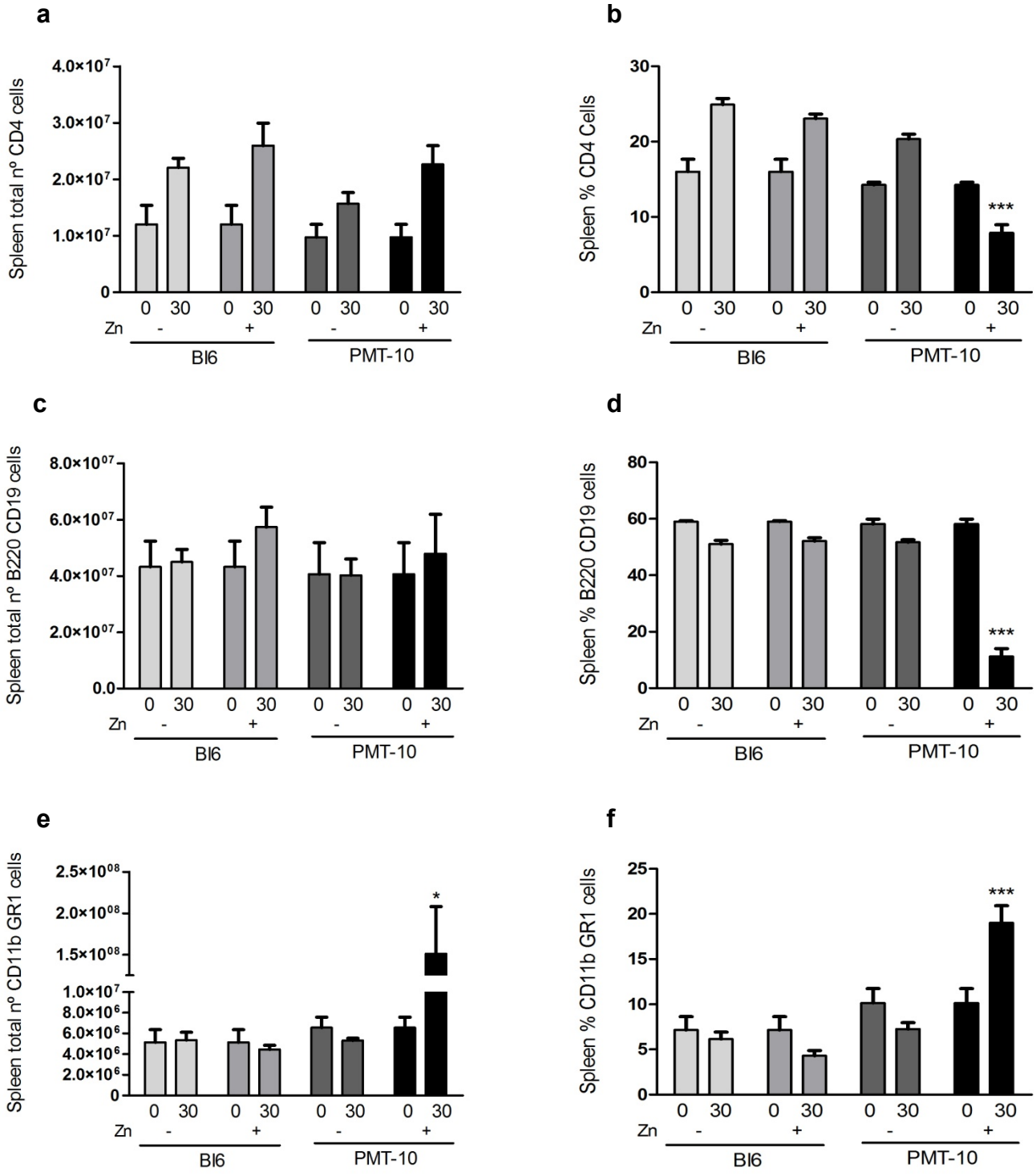


Figure 10. IL-10 over-expression in PMT-10 mice affects the proportion of spleen cells population. At day 30, single cell suspensions from spleens from WT and PMT-10 mice exposed or not to Zn were prepared, stained with specific antibodies for B220, CD3, CD4, CD8, CD11b, CD19 and GR1 and analyzed by flow cytometry. The total number of cells was determined using the Cell Counter (Countess™ Invitrogen). Data from one experiment representative of three, with mean ± SEM from 5-6 mice per group, one way ANOVA * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

3.2.2 IL-10 over-expression impacts hematopoiesis in the BM

Since we observed alteration on the cellular profile in the spleens of mice over-expressing IL-10, we next hypothesized that IL-10 could be affecting the hematopoietic process in the BM. To evaluate this, we measured by flow cytometry, the expression of B220, CD11b, CD19 and GR1 populations in the BM after 30 days of IL-10 over-expression in the PMT-10 mice. We found a similar profile to the observed in the spleen, characterized by a sharp decrease in the B cell subset and an increase in the macrophage/ granulocyte population upon IL-10 over-expression (figure 11).

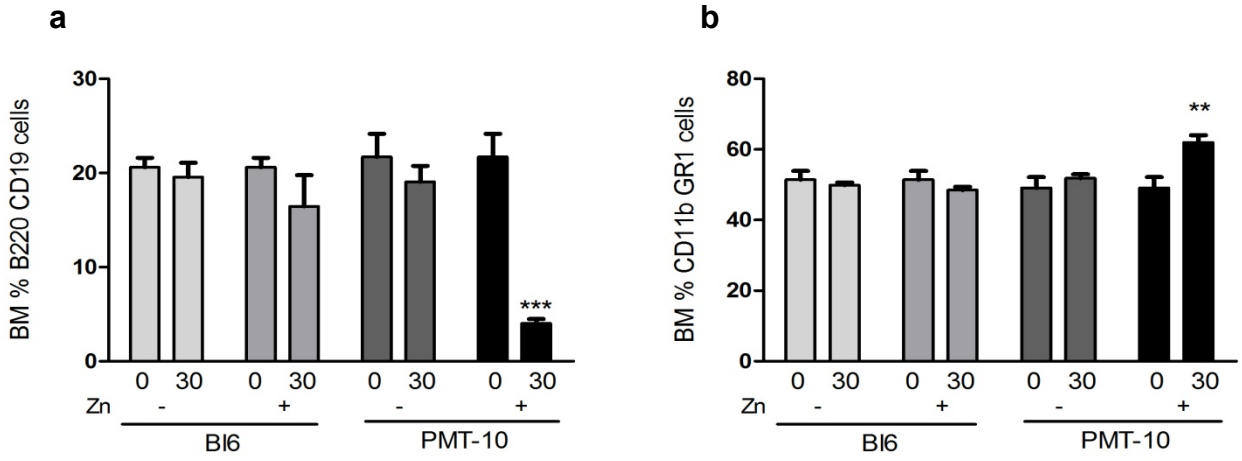


Figure 11. IL-10 over-expression alters the proportions of cell populations in the BM. At day 30 of IL-10 over-expression in PMT-10 mice BM from all groups was harvested and the single cell suspensions were stained with specific antibodies for B220, CD111b, CD19 and GR1. The analysis was performed by flow cytometry and the total cell number determined using the cell counter (Countess™ Invitrogen). Data of one experiment representative of three, with mean ± SEM from 4-6 mice per group from one experiment, one way ANOVA * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

To further investigate these results, we next investigated whether the composition of early hematopoietic precursors in the BM was altered by IL-10 over-expression. In this sense, we harvested the BM of PMT-10 mice over-

expressing or not IL-10 at day 30 of Zn exposure. Single cell suspensions from BM were marked for lymphoid precursors with specific antibodies (B220 and CD19). To evaluate myeloid precursors, we performed an enrichment of HSCs by magnetic separation and marked the cells with specific antibodies (SCA1, cKIT, Fcγ and CD34). Our findings revealed that PMT-10 mice over-expressing IL-10 present a shift from the cKIT population towards the SCA1 population (figure 12). These results have been also seen in transgenic mice positive for the mutation V617F with a phenotype resembling PV [146]. Moreover, a severe reduction of the lymphoid precursors ($B220^{low}CD19^{+}$) (figure 13a) and MegE progenitors ($FcγR^{low}CD34^{-}$) and an expansion of the GMP ($FcγR^{high}CD34^{+}$) were observed (figure 13b).

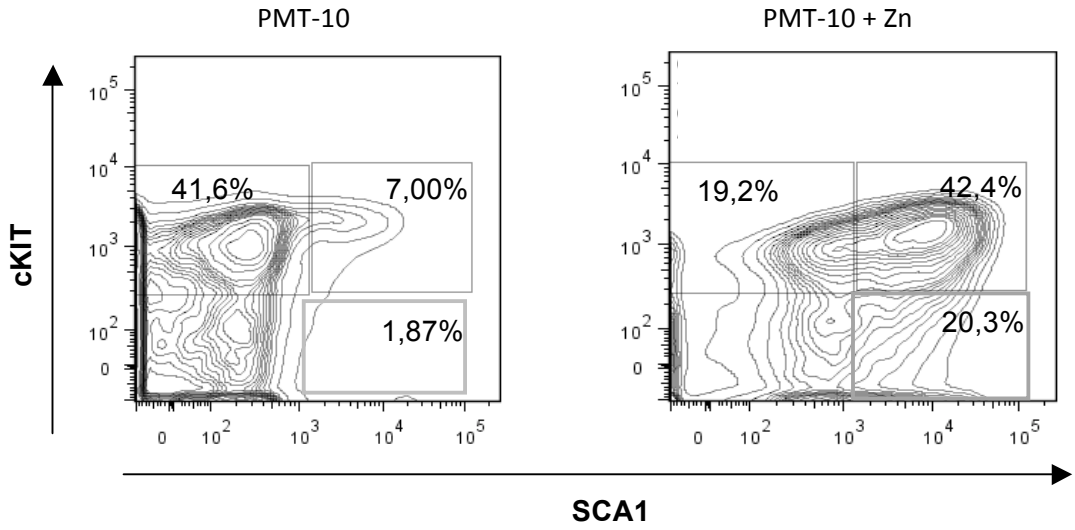


Figure 12. SCA1 population increases in PMT-10 mice over-expressing IL-10. PMT-10 mice exposed or not to Zn were analysed for immature cells from the myeloid lineage. At day 30 of IL-10 over-expression, single cell suspensions from BM were prepared, enriched for undifferentiated cells by magnetic separation and stained with surface markers (IL-7Rα, CKIT and SCA1) for flow cytometry analysis of immature cells from the myeloid lineage. Data represents the mean from 3-5 mice per group from one experiment.

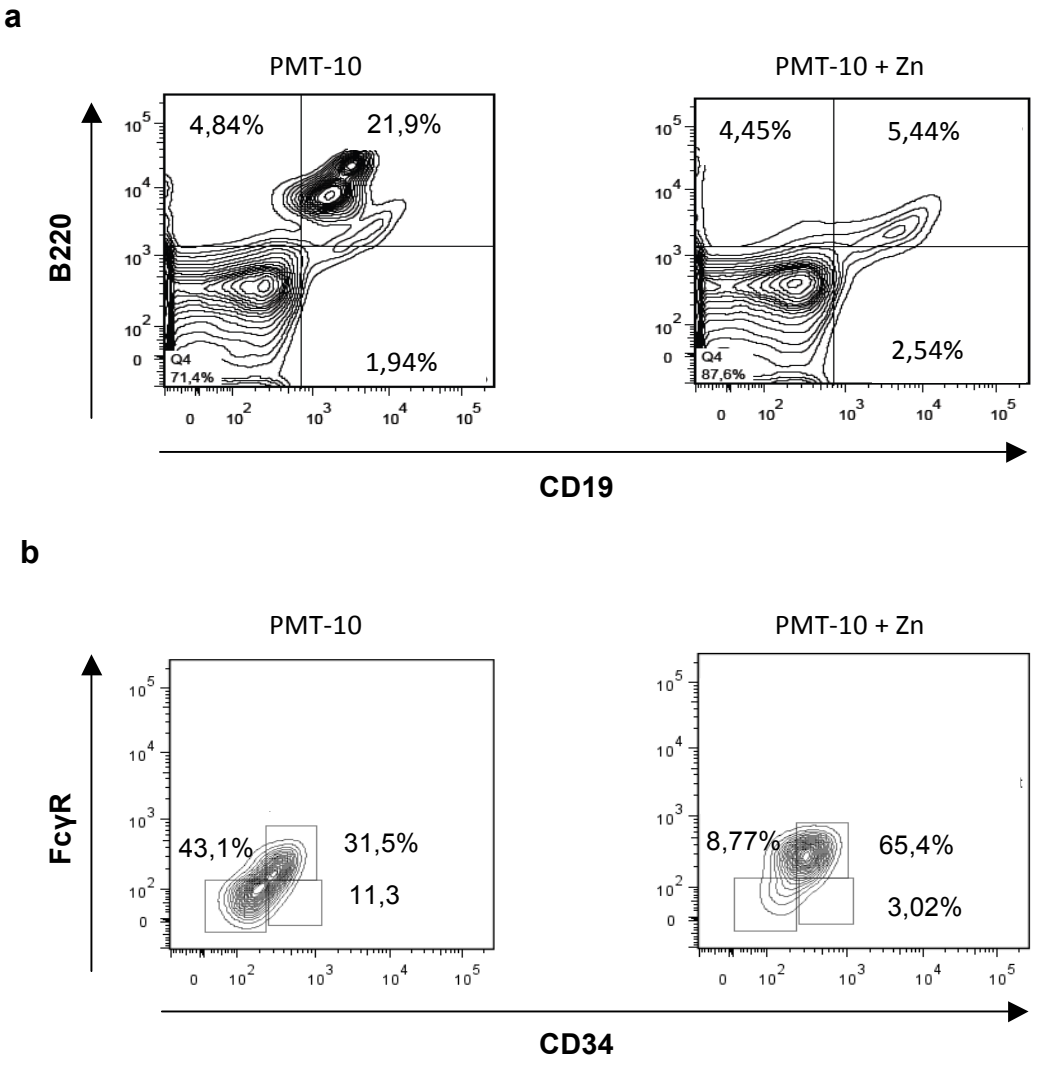


Figure 13. IL-10 over-expression in PMT-10 mice alters the normal differentiation of myeloid and lymphoid population in the BM. At day 30 of IL-10 over-expression in PMT-10 mice, BM from both groups was harvested and analyzed by flow cytometry for lymphoid precursors - B220low/CD19+ cells (a). In parallel, HSCs were enriched by magnetic separation and analyzed by flow cytometry for common myeloid progenitors – Lin-SCA1-cKit+ (b). The total number of cells was determined using the Cell Counter (Countess™ Invitrogen). Data represent the mean from 3-6 mice per group from one experiment.

3.2.3 IL-10 over-expression leads to alterations of the BM structural organization

Because we observed that IL-10 over-expression led to profound alterations in the hematopoietic process and in the proportion of cellular populations in the BM, we next investigated whether the overall organization of this organ was affected by IL-10. For this, histological sections from wild type or PMT-10 mice exposed or not to Zn were prepared and stained with HE. As shown in figure 14, the BM of PMT-10 mice over-expressing IL-10 presented an altered structure of BM with a striking disappearance of megakaryocytes, indicating that IL-10 was affecting the hematopoietic process. The decrease cellularity and the disappearance of megakaryocytes, together with the massive decrease of B cells and increased myeloid population in the BM, suggest the impairment of hematopoiesis. Furthermore, these events are in line with the occurrence of a extramedullary hematopoiesis at the spleen level. These results are in line with the MPD profile since a decrease BM cellularity with special decline of erythropoiesis and megakaryocytopoiesis is one the characteristics from patients suffering from MPD [114, 119-121, 144, 145], suggesting that IL-10 over-expression may influence normal hematopoiesis in a very similar way to MPD.

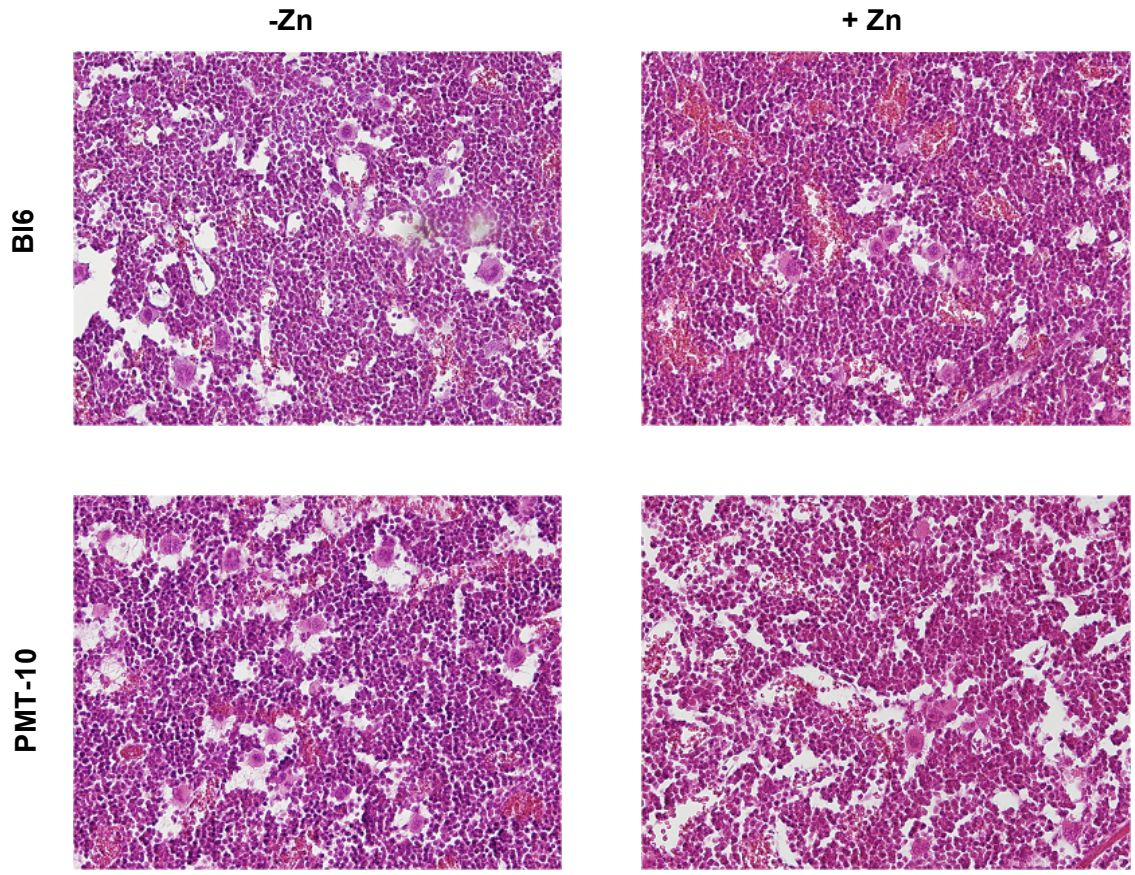


Figure 14. Structural disorganization of BM of IL-10 over-expressing PMT-10 mice. Femurs from all groups – WT and PMT-10 mice exposed or not to Zn, were removed and left in formaldehyde 3,7% for one week. After decalcification histological analysis was performed by staining with HE (200x). Each image shows one mouse representative of each.

3.2.4 IL-10 over-expression affects the lymphoid and myeloid populations from the blood

So far, we have shown that IL-10 over-expression caused cellular alterations both in the BM and the spleen. Since the blood is used by the different cellular subsets to move from one compartment to another, the next step was to evaluate if the blood cellularity was being affected by the high levels of IL-10. To test this, peripheral blood from all groups –, was drawn from the tail/eye. Hematocrit analysis was performed showing that the red blood cells (RBC) percentage in PMT-10 mice over-expressing IL-10 decreases overtime (figure 15a). Furthermore, the peripheral blood was analysed by flow cytometry for the major leukocytes subsets, showing very similar data to the one seen in

the spleen and BM (figure 9 and 10), characterized by a decrease of the percentage of B cell population and an increase of granulocytes/macrophage population (figure 15b and 15c).

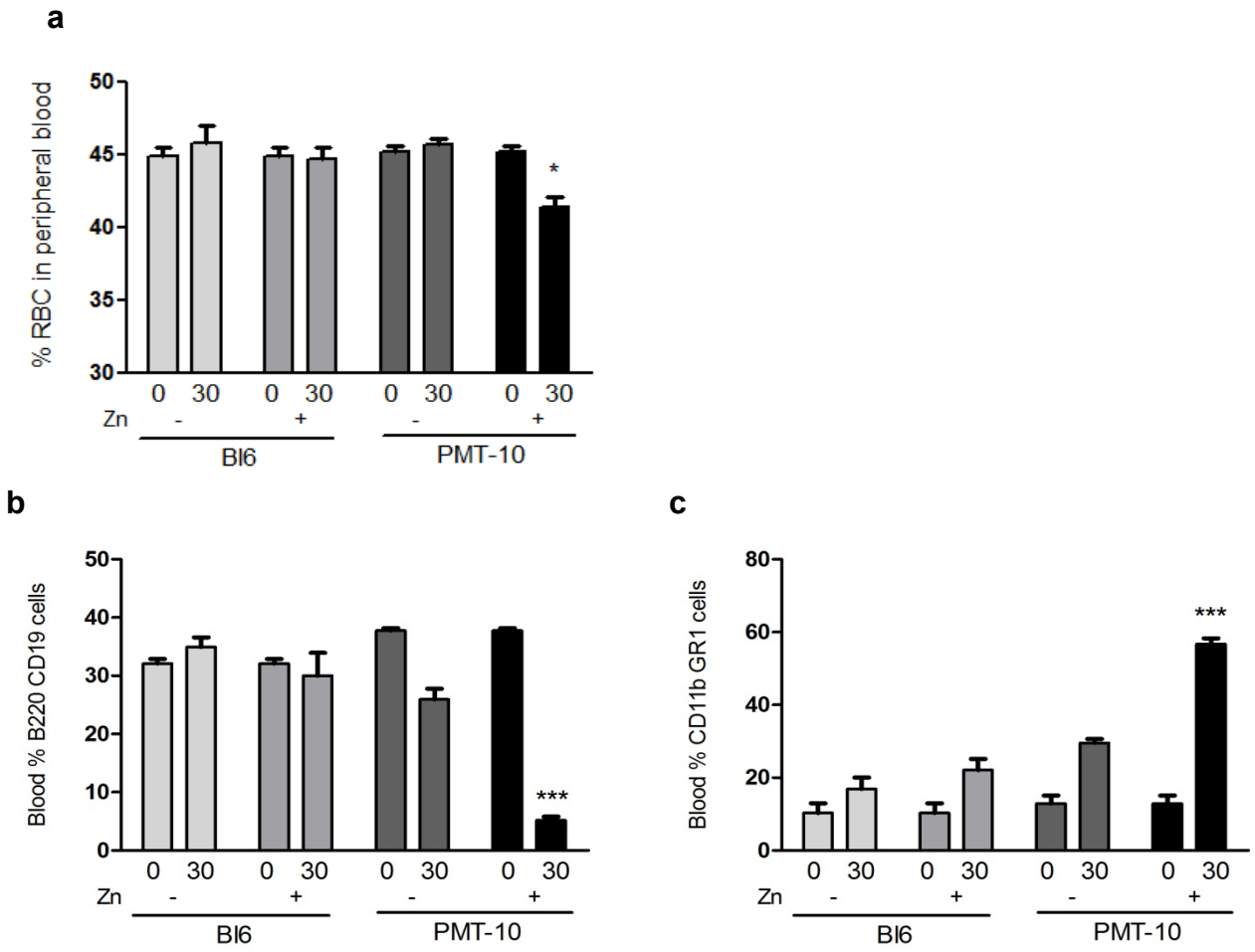


Figure 15. IL-10 over-expression in PMT-10 mice leads to alterations of the normal populations in the PB. At day 30 of IL-10 over-expression in PMT-10 mice, PB from the eye/tail was drawn from all groups and analysed by hematocrit (a) and flow cytometry for the lymphoid (b) and myeloid populations (c). Data from one experiment representative of three, with mean ± SEM from 5-6 mice per group from one experiment, one way ANOVA* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.3 Reversibility of IL-10 over-expression

3.3.1 Withdrawal of IL-10 to normal levels led to the reposition of cellular populations in the spleen, BM and blood

Taken together, the data presented in part 3.2 suggest a possible role for IL-10 as a regulator of the hematopoietic process, since over-expression of this cytokine in a mouse model leads to inhibition of lymphoid lineage, and increased myeloid lineage differentiation in the BM of adult animals. PMT-10 mice over-expressing IL-10 develop a splenomegaly, accompanied by an increase of the total cell number mainly composed by cells of the myeloid lineage. BM from these animals present an impairment in hematopoiesis, that leads to a cellularity decrease. These features result in a specific phenotype that resembles the one seen in MPD patients [114, 119-121, 144, 145]. Nevertheless several questions remain open, such as the evaluation of the plasticity of our mouse model in recovering, both at the macroscopic and the functional levels, from the phenotype induced by IL-10 over-expression.

We now want to explore our findings further, at the cellular and molecular levels, to clarify how IL-10 can shift hematopoiesis towards the myeloid lineage. Currently we are studying the plasticity of the organism when exposed to IL-10. To do so, we are performing experiments by exposing the animals to Zn for 30 days followed by 15 days without Zn and repeat this cycle 3 or more times. At the end of each exposure or resting period, a group of animals will be sacrificed and the spleens, BM and blood will be harvested for cell population analysis by flow cytometry and tissue organization by histology (figure 16).

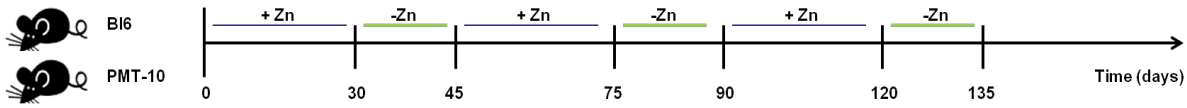


Figure 16. Experimental layout. The plasticity of the system will be addressed by performing analysis of several parameters in various time points.

PMT-10 mice were induced to over-express IL-10 during 30 days. At day 30, the Zn feeding that induces IL-10 over-expression was discontinued, and 15 days were given to the mice to recover. At various time points, PB was collected to measure the levels of IL-10 by immunoassay (figure 17) and the percentage of RBC (data not shown). As showed before, the levels of IL-10 in PMT-10 mice achieved a plateau 15 days after the beginning of the Zn administration, while the levels in the other groups remained below detection level. Furthermore, 5 days after the end of induction, the levels of IL-10 in PMT-10 mice was no longer detected (figure 17).

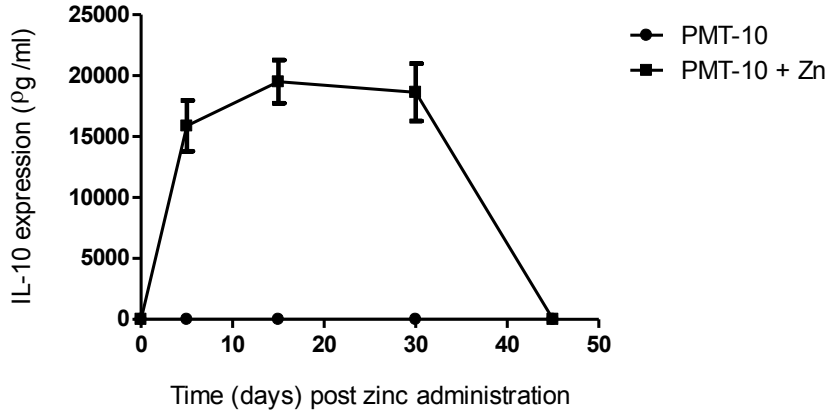
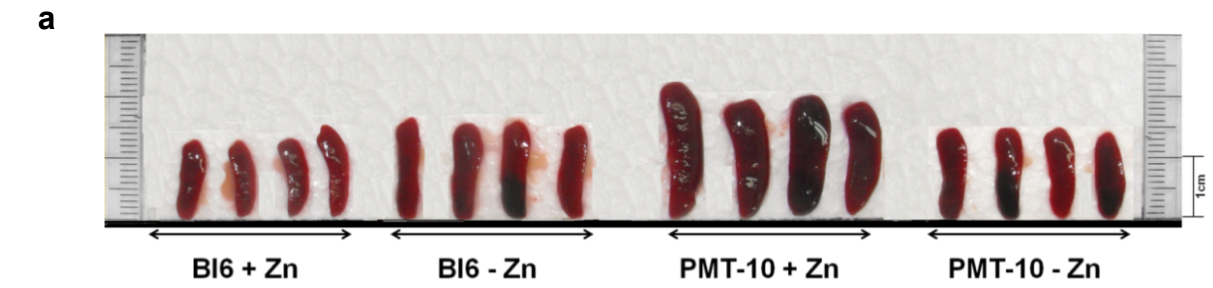
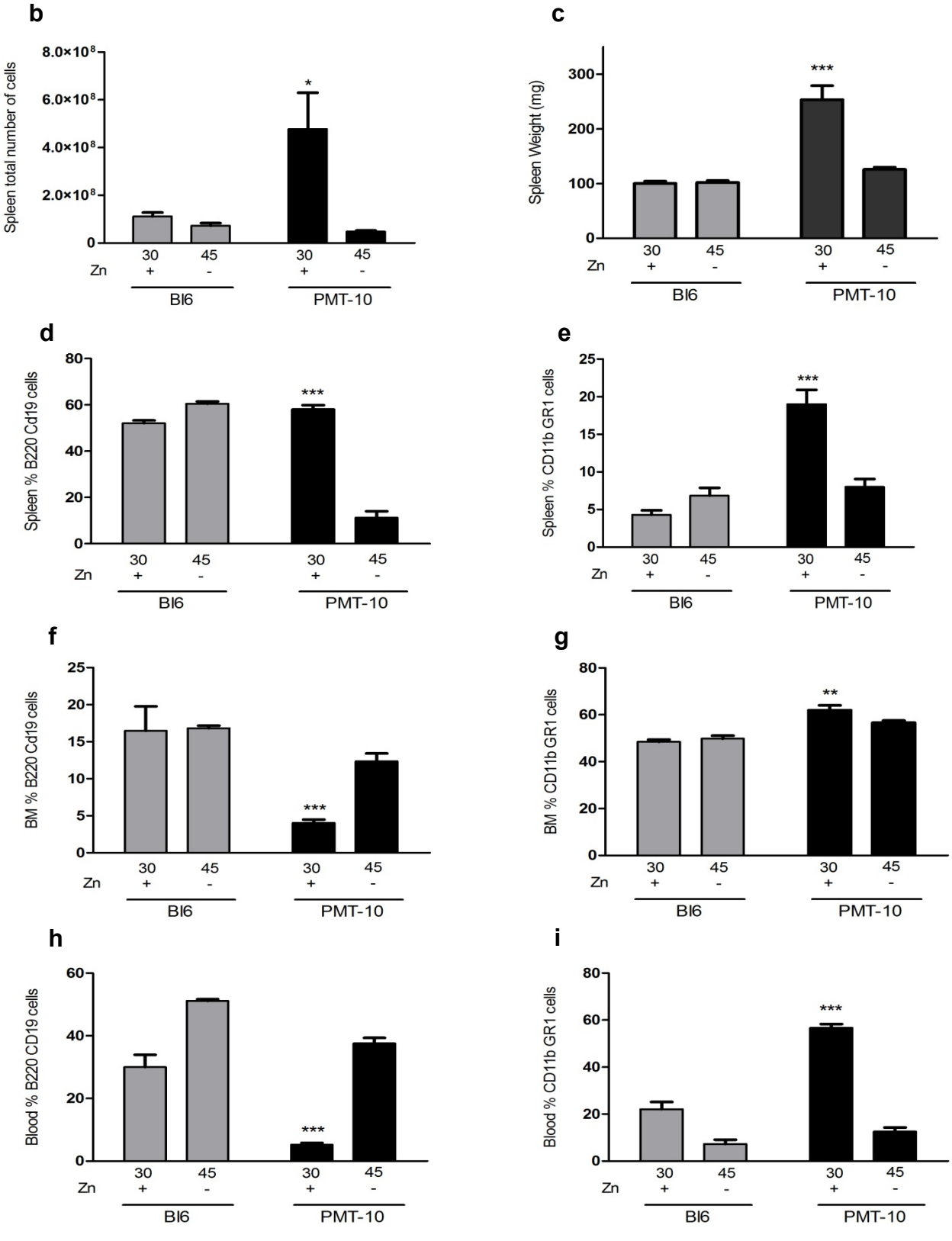


Figure 17. IL-10 kinetics in PMT-10 mice after the end of Zn administration. PMT-10 mice were induced to over-express IL-10 for 30 days with a Zn solution. At day 30, this induction was discontinued, giving the normal water to the 2 groups previously treated with Zn – WT and PMT-10 mice. 5 days after, IL-10 concentration was determined in the serum of the induced PMT10-mice and all control groups – WT exposed or not to Zn and non-treated PMT-10 mice, by ELISA. Data represent the mean ± SEM from 6 mice per group.

Spleens from PMT-10 and WT mice exposed to Zn for 30 days, were harvested 15 days after the cessation of Zn feeding and compared (day 45). As show in figure 18a, the PMT-10 mice spleen returned to its normal size.


a
Bl6 + Zn
Bl6 - Zn
PMT-10 + Zn
PMT-10 - Zn
1cm


b
Spleen total number of cells
8.0×10⁸
6.0×10⁸
4.0×10⁸
2.0×10⁸
0
*
30 45 30 45
Zn + - + -
Bl6
PMT-10
c
Spleen Weight (mg)
300
200
100
0
***
30 45 30 45
Zn + - + -
Bl6
PMT-10
d
Spleen % B220 Cd19 cells
80
60
40
20
0
***
30 45 30 45
Zn + - + -
Bl6
PMT-10
e
Spleen % CD11b GR1 cells
25
20
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10
5
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30 45 30 45
Zn + - + -
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BM % B220 Cd19 cells
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30 45 30 45
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80
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Blood % CD11b GR1 cells
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60
40
20
0
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30 45 30 45
Zn + - + -
Bl6
PMT-10

Figure 18. IL-10 withdrawal leads to recover of normal phenotype. After 15 days of the end of Zn administration the PMT-10 mice, previously exposed to IL-10 over-expression for 30 days, spleens from all groups were removed and compared for its size (a), total number of cells (b) and weight (c). Single cell suspensions from spleen, BM and blood were analysed by flow cytometry using specific antibodies (B220, CD3, CD4, CD8, Cd11b, CD19 and GR1) (d-i). The total number of cells was determined using the Cell Counter (Countess™ Invitrogen). Data represent the mean ± SEM from 4-6 mice per group from one experiment, one way ANOVA * $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

Furthermore, this size regression of the spleen was accompanied by a decrease of the total number of cells, returning to its normal values from approximately 100 million cells (figure 18b). The spleen weight of previously PMT-10 mice over-expressing IL-10 also decreased (figure 18c). Moreover, all the different cell populations of the spleen, BM and blood returned to its normal values (figure 18d-i), including the T cell populations and RBC (data not shown).

3.3.2 Withdrawal of IL-10 to normal levels led to restoration of spleen and BM histology

Samples from spleens collected at day 45 from Bl6 and PMT-10 mice previously exposed to Zn were processed for histological studies by staining with HE. PMT-10 mice exposed to IL-10 over-expression that presented a tissues structural disorganization from the spleen (figure 8), recovered its structural organizaiton15 days after the cessation of Zn exposure, being possible to distinguish again the white and the red pulp (figure 19). Moreover, the giant cells morphologically resembling megakaryocytes that appeared in great number in PMT-10 mice over-expressing IL-10, were reduced to absent at day 45. Samples from the BM of PMT-10 mice presented also a recovery of the normal populations (figure 19).

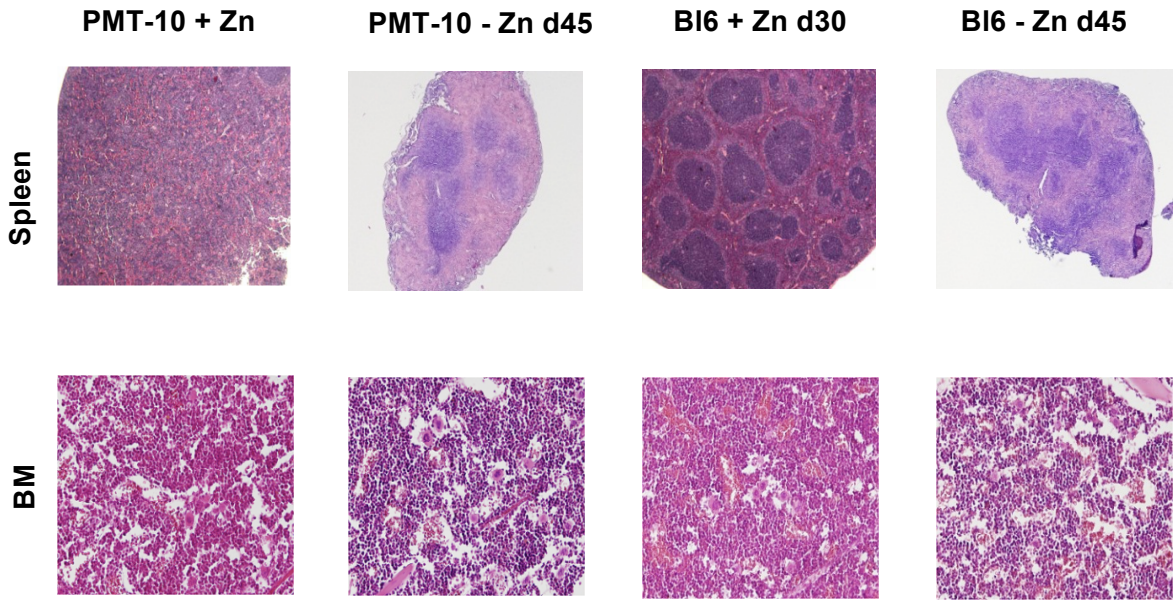

Figure 19. Withdraw of IL-10 in PMT-10 mice leads to the recovery of normal structural organization of the spleen and BM. At day 45, 15 days after the end of Zn exposure, spleens and BM from WT and PMT-10 mice were harvested, and left in formaldehyde 3,7% for 2 days or one week, respectively. After BM decalcification the samples were histological analyzed by HE. Each image shows one mouse representative of each group.

Altogether, our results suggest that IL-10 over-expression for 30 days in PMT-10 mice impacts hematopoiesis. These features, seen in PMT-10 mice over-expressing IL-10 and characterized by a splenomegaly, increased total cell number in the spleen due to the proliferation of cells from the myeloid lineage, increased numbers of megakaryocytes in the spleen, structural disorganization of the spleen and loss of cellularity of the BM with special incidence in the B cell and megakaryocyte/erythrocyte population are seen in a very specific group of hematopoietic disorders, the MPDs. However, our data so far, does not allow us to determine if IL-10 over-expression has a reversible or irreversible effect in the plasticity of the system.

4. DISCUSSION

Every day billions of blood cells reach maturity, each derived from HSC through process of differentiation and proliferation that origin the lymphoid and myeloid lineage, maintaining homeostasis and guaranteeing protection against pathogens [1-3]. Differentiation of HSCs occurs in fetal liver and in BM throughout adulthood, under the control of signals delivered by stromal microenvironment [1-4]. These signals regulate the proliferation, the survival and the differentiation of HSCs by activating genetic programs that determine the specification of the precursors along the different lineages, with loss of potential for differentiation into other cell fates [1-4, 6-11]. Deregulation of the hematopoietic process often leads to fatal diseases, such as immunodeficiencies, leukemias and MPDs [51, 57, 95-97]. Therefore, the understanding of the molecular mechanisms that regulate hematopoiesis is of great importance. Several lines of evidence implicate interleukins, IFNs and colony stimulating factors in hematopoiesis [35, 51, 52, 54, 62, 63, 153]. Each of these factors functions alone or in combination with the others. Moreover, the direct action of these factors in a cell lineage can influence indirectly other lineages [9, 43, 154-156].

The influence of several cytokines, such as IL-3, IL-7 or GM-CSF, in hematopoiesis has been well reported over the years [31, 51, 53, 54, 153, 154, 156] but the role of IL-10 in this process is still awaiting clarification. So far, the presence of IL-10 has been implicated in several hematopoietic disorders, such as diffuse B cell lymphoma (DBCL) and non-Hodgkin's lymphoma [142, 157]. This possible involvement raises several questions, including the detailed understanding of the impact of IL-10 on cell differentiation and proliferation. In this context, a mouse model able to transiently over-express IL-10 under a tight control presents itself as an interesting tool to study the impact of IL-10 in hematopoiesis. PMT-10 mice are such model, where IL-10 over-expression is induced after Zn administration.

Our results, obtained by comparing PMT-10 under Zn administration to control PMT-10 or to Bl6 mice fed with Zn or not, demonstrate that high levels of IL-10 may impact normal hematopoiesis. By characterizing in detail the effect of IL-10 over-expression in PMT-10 mice, we found that in this situation they presented a pronounced splenomegaly not seen in the remaining groups (figure 7a), accompanied by an increase of total spleen cell numbers and spleen

weight (figure 7b and 7c). A structural disorganization of the spleen of PMT-10 mice over-expressing IL-10 was also observed (figure 8a), with the appearance of giant cells, morphologically resembling megakaryocytes (figure 8b). These cells were found in various stages of differentiation, including early stages of development, suggesting that they were being produced in the spleen. These first evidences, characterized by splenomegaly and increase in the number of megakaryocytes in the spleen of PMT-10 over-expressing IL-10, called our attention since they resemble the phenotype exhibited by MPD patients [114, 119, 121, 123, 127]. Moreover, this same profile has also been reported in mouse models of MPD, namely those expressing the $JAK2^{V617F}$ mutation [144, 145]. Whether PMT-10 mice over-expressing IL-10 represent a novel model of MPD remains to be clarified.

Secondly, we need to clarify whether extramedullary hematopoiesis is occurring. For that, we started by investigating in detail the impact of high IL-10 levels in the cells of the myeloid and lymphoid lineages. In the spleen of PMT10 mice over-expressing IL-10, we observed a substantial increase in myeloid cells (CD11b+GR1+) with a corresponding decrease in B and T-lineage cells (figure 10). Moreover, we observed that the decrease seen in B and T-lineage cells was not due to a drop of its total cell number but to a massive increase of total myeloid cell numbers, which altered the normal ratio between these subsets. Although an increase of the total number of myeloid cells was observed, one question remained. Were the cells from the myeloid lineage proliferating in the spleen? Or were these cells accumulating in the spleen due to defect in their homing/egress? Answers to these questions are essential to prove or not the occurrence of extramedullary hematopoiesis and therefore to conclude whether IL-10 over-expression I slinked to MPD.

To answer the first question, we started evaluating the different leukocyte subsets in the BM.

We found that, similarly to the changes seen in spleen, the BM presented a sharp decrease of the B cell population whereas cells from the myeloid lineage suffered an increase (figure 11). To further investigate our findings, we next addressed if the IL-10 induced differences seen in the proportions of B cells and myeloid cell in the BM resulted of alterations of the hematopoietic process. Investigation of the composition of early hematopoietic precursors revealed that

30 days after induction of IL-10 expression in PMT-10 mice, a significant increase of the SCA1 and SCA1/ cKIT positive cells was observed in the BM (figure 12). It has been reported that SCA is a marker highly expressed in primitive HSCs whereas cKIT is expressed not only in HSCs but also in the common progenitors [146]. A shift towards the SCA positive population observed in PMT-10 mice over-expressing IL-10 indicates that hematopoiesis in the BM was being affected in a very early step of differentiation (figure 12).

Moreover, the remaining cells that followed the pathway of differentiation, that is the cKIT positive cells, suffered a bias towards the myeloid lineage. These alterations resulted in a severe reduction in lymphoid precursors (B220lowCD19+) (figure 13a) and an expansion of CMP in the BM of PMT-10 mice over-expressing IL-10 (figure 13b). Interestingly, the different myeloid precursors were not affected equally, being the differentiation into MegE progenitors inhibited whereas the differentiation into GMP was stimulated, resulting in a granulocyte/macrophage proliferation. The phenotype observed in BM after IL-10 over-expression, was similar to that seen in human patients and in mouse models of MPD [114, 119-121, 123, 144, 145].

A structural analysis of the BM of PMT-10 mice over-expressing IL-10 showed interesting results, revealing a decrease of the BM cellularity with a visible decline of erythropoiesis and megakaryocytopoiesis (figure 14). These features are suggestive of BM aplasia, a condition characterized by a gradual decrease of cell count resulting in the production of fewer blood cells, including a sharp drop of RBC and white blood cells [158]. Thus, the increase of myeloid cells seen in the spleen may not strictly be due to a stimulation of proliferation of the myeloid lineage in the BM, but due to extramedullary hematopoiesis at the spleen level.

In future, we will explore our findings further, by evaluating the occurrence of a local cell proliferation in the spleen of Zn-exposed PMT-10 through bromodeoxyuridine (BudR) quantification. Moreover, to investigate whether the homing/egress of cells to and from the spleen mice over-expressing IL-10 is altered, we will evaluate the expression of chemokines involved in the cell displacement from one organ to another, by analysing their expression profile by RT-PCR.

Although we have seen that, in the BM, IL-10 over-expression led to a shift of hematopoiesis towards the myeloid lineage, we have not yet clarified the mechanisms by which IL-10 impacts the hematopoietic process. Is IL-10 acting directly or indirectly via other factors? (figure 20) CMP differentiation is regulated by several factors, such as IL-3, GM-CSF and erythropoietin [6]. Any influence in the expression of these cytokines, whether negative or positive, is expected to have consequences in the normal course of hematopoiesis. IL-3 acts synergistically with other cytokines to promote the differentiation of the myeloid progenitors [54, 55]. Several studies demonstrate that numerous transcription factors are induced by cytokines in the microenvironment where lymphopoiesis is occurring to control the expression of B cell–specific genes. E2A induces expression of EBF and, together, these two transcription factors activate Pax-5, whose expression marks commitment to the B cell lineage [29, 46, 159]. IL-7 has an important role in the expression of EBF in CLP, whereby its blockade leads to an impairment of B cell differentiation [31, 53]. In Pax-5 deficient mutants, B cell differentiation is also blocked, albeit at a later stage than in EBF mutants, and precursors retain the ability to differentiate into other hematopoietic lineages [47]. It is possible that IL-10 influences hematopoiesis indirectly whereby the cytokines and transcription factors mentioned above are good candidates for the role of intermediate molecules. IL-10 may be acting through IL-3, inducing its expression and consequently leading to a proliferation of the myeloid lineage. On the other hand, IL-10 can also be blocking the expression of IL-7, promoting the proliferation of the myeloid lineage indirectly, by blocking the differentiation of CLP. A synergistic action between IL-10 and IFNα is also possible, given the ability of IFNα to promote differentiation of HSCs [65]. The mechanism by which IL-10 impacts hematopoiesis is a topic that we also want to address in the future. Using highly enriched HSCs isolated by cell sorting and differentiated in vitro in stromal cell-free cultures, in the presence or absence of IL-10, we would be able to determine the impact of IL-10 over-expression in the differentiation of different cell lineages in vitro. These assays would allow us to assess whether IL-10 affects hematopoiesis by directly acting on uncommitted precursor cells. If we observe that IL-10 is acting indirectly, the impact of candidate cytokines mediating this effect will be assessed by using available knock-out mice, antibodies to those proteins or by

the addition of recombinant cytokines. We also plan to measure the presence of these cytokines in the serum of PMT-10 mice over-expressing IL-10 and compare the obtained values to those of control animals.

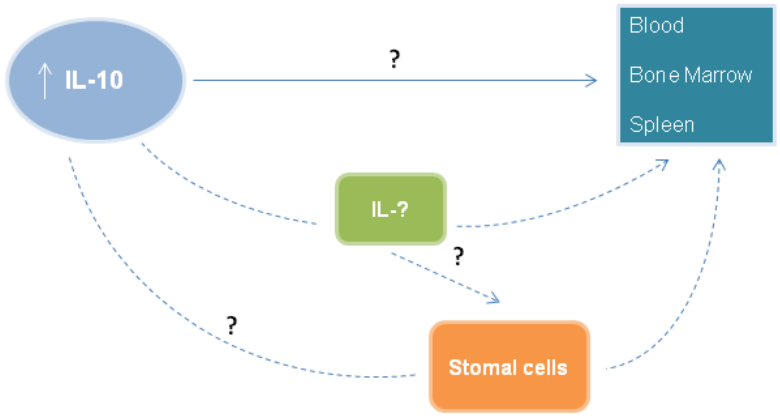

Figure 20. Representative scheme of possible mechanisms of IL-10 impact in hematopoiesis.

As already mentioned, the phenotype revealed in PMT-10 mice upon Zn exposure is similar to that observed in MPD. Indeed, the splenomegaly, increased total spleen cell numbers, structural disorganization and increased number of megakaryocytes in the spleen, are all features observed in mouse models and humans patients of MPDs. Interestingly, the alterations seen in the BM, namely the hematopoiesis impairment, loss of cell cellularity, decreased erythropoiesis and consequently decreased % of B cells and RBC, are all in line with a MPD. In addition, blood analysis revealed similar results to the ones seen in the spleen and BM, characterized by a decrease on percentage of B cells population and an increase on myeloid cells in PMT-10 mice over-expressing IL-10 (figure 15b). Moreover, a decrease in the % of RBC in PMT-10 over-expressing IL-10 was observed (figure 15a). Accordingly to the WHO guidelines, the patients suffering from PV present an increased % of RBC while patients suffering from ET or PMF present a normal or small decrease % of RBC [114]. The decreased value of RBC seen in PMT-10 mice over-expressing IL-10 can be related to the increase % of platelets (data not shown) by an

alteration of normal cellular proportions, and not only to the impairment of erythropoiesis. Since megakaryocytes are the platelets precursors, the increase in the number of megakaryocytes in PMT-10 mice over-expressing IL-10 might result in an increase platelet count, explaining these results. It will therefore be of utmost importance to now quantify a series of parameters in PMT-10 mice, such as total and differential counts of white blood cells, RBC and platelets including the mean globular volume and the mean platelet volume. All these parameters were shown to be altered in a MPD scenario.

MPD are frequently caused by mutations on the JAK2 gene, which are present in 90% of patients with PV and 50% of the patients suffering from ET or PMF [114, 129, 133, 143, 147]. Based on the epidemiology of MPD and on the phenotype observed for IL-10 over-expressing mice, we hypothesize that increased IL-10 may associate with susceptibility to MPD, explaining the 10 - 50% of cases unrelated to the JAK2V617F mutation. Therefore, PMT-10 mice are of potential interest as a novel model to study the molecular mechanisms leading to MPD, in the context of non-mutated JAK2. Since IL-10 has been recently shown to induce abnormal JAK2 activation in the context of DBLC [142], we have accessed whether JAK2 hyper-activation underlies the development of MPD in the PMT-10 mice. To study this hypothesis, we are currently comparing by Western Blot the levels of JAK2 phosphorylation in BM cells from PMT-10 mice over-expressing or not IL-10.

To study the plasticity of the PMT-10 model in recovering from IL-10 exposure, we have exposed the animals to Zn for 30 days followed by 15 days without Zn administration, stopping the induction of IL-10 (figure 17). A regression to normal size from the spleens of PMT-10 mice that previously presented a splenomegaly was observed (figure 18a). Moreover, all changes that accompanied the splenomegaly disappeared, occurring a regression of weight and total number of cells of the spleens from PMT-10 mice previously exposed to Zn (figure 18b and 18c). The different cell populations, including the % of RBC, returned to normal in all organs previously affected namely the spleen, BM and blood (figure 18f-i). However, there are some questions that need to be further investigated. Firstly, even though the spleen has recovered his structural organization, are the different cell populations in their normal location? Secondly, are the cells functional after these alterations? Thirdly, how

plastic is this system? These are questions that we would like to address in the future.

Regarding the structure of the spleen, we will analyse if it returns to normal after the end of the Zn induction. To do so, we will perform a detailed analysis of cellular composition and distribution, using specific antibodies to trace B and T cells, and immunohistochemistry to observe the localization of marginal zone B cells and follicular B cells. In parallel, and to address if the spleen function is recovered, we will infect animals intravenously with *Streptococcus pneumonia,* measuring the progression of the bacterial load over time and the life span of the infected PMT-10 mice. Finally, and in what concerns the third question, we are currently addressing more thoroughly the system plasticity, by exposing the animals to Zn for 30 days followed by 15 days without Zn, and repeating this cycle 3 or more times. This will provide evidence on how transient is the effect of IL-10 over-expression. Of note, we have recent data showing that PMT-10 over-expressing IL-10 do not survive for more than 160 days, presenting a splenomegaly even more notable than day 30 and a gradual cellular loss in the BM (data not shown).

In summary, the work presented here highlights two novel findings: that high IL-10 amounts impacts hematopoiesis, promoting the differentiation of myeloid cells and that IL-10 may be involved in the development of MPD in a context of no JAK2 mutations. Although further mechanistic studies are needed to explain these findings, this thesis opens many new questions and avenues of research that are worth investigating. Furthermore, if needed a parallel between the effects of IL-10 over-expression and MPD is in place, the elucidation of these mechanisms, using PMT-10 mice, may lead to the development of new approaches to the tackle MPD.

5. CONCLUSION

The presented work investigates for the first time the role of in vivo IL-10 over-expression in hematopoiesis. We showed that IL-10 over-expression impacts the hematopoietic process by impairing the development and promoting the differentiation of the myeloid lineage. These features acquired with IL-10 over-expression led to:

- a splenomegaly, structural disorganization and increased number of megakaryocytes in the spleen, with an associated decrease of cells from lymphoid lineage.
- a decrease of the BM cellularity with a visible decline of erythropoiesis and megakaryocytopoiesis.
- a shift of the cKIT population towards the SCA positive population.
- the stimulation of GMP differentiation.

Understanding the molecular mechanisms that regulate hematopoiesis is important, taking into account that this process is responsible for the production of all blood cells and that its deregulation leads to fatal diseases. With this work we place IL-10 as one of the mediators of the hematopoietic process with possible implications with disease.

The phenotype observed in PMT-10 mice over-expressing IL-10 show some similarities to the one seen in patients and mouse models of MPD. To establish PMT-10 mice as a potential model for MPD, several factors need to be further clarified. If this proves to be the case the elucidation of the molecular mechanisms underlying IL-10 action, using PMT-10 mice, may lead to the development of new approaches to study and tackle MPD.

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