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Escola de Medicina

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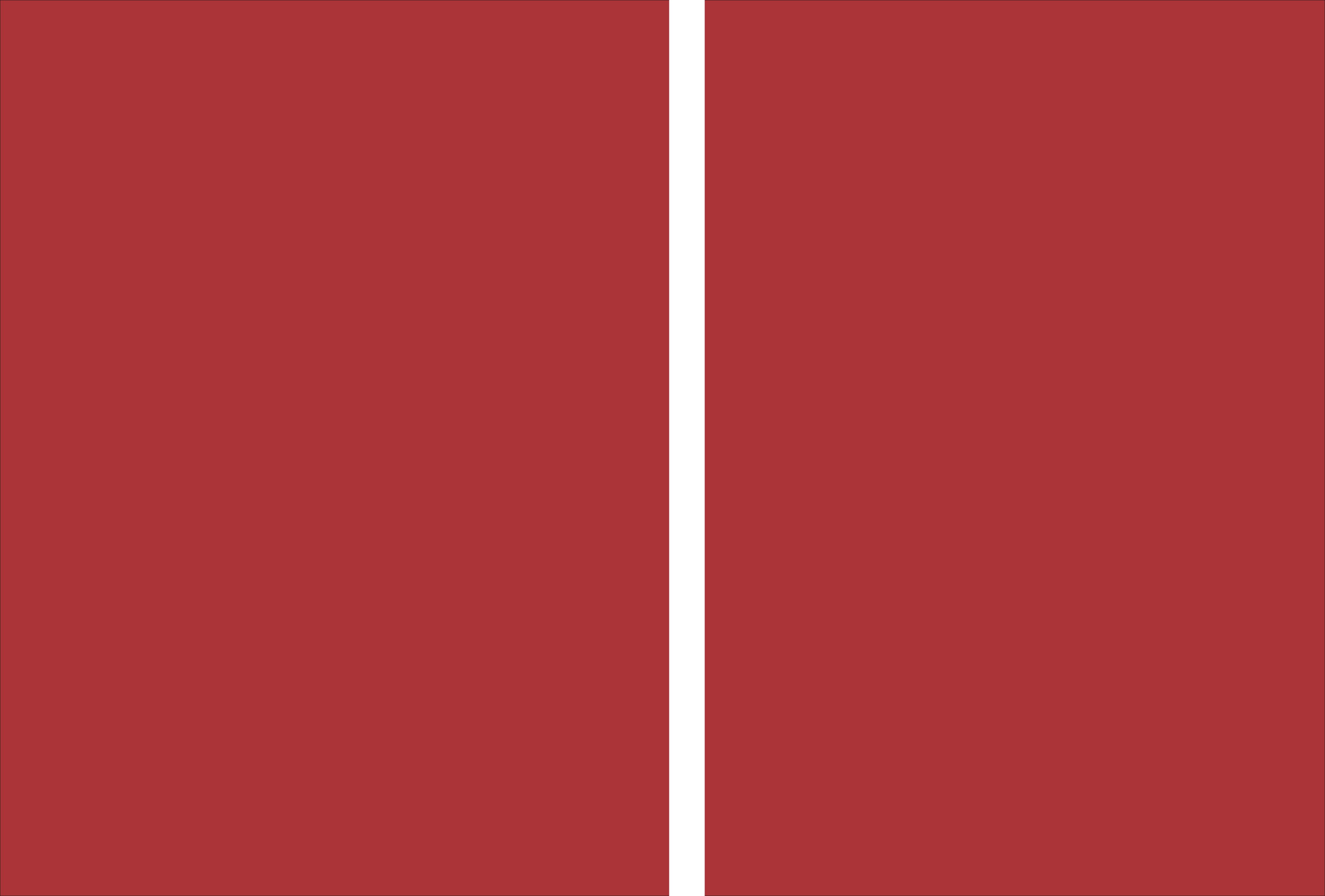
Impact of Regulatory Cytokines in Hematopoiesis

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Impact of Regulatory Cytokines in Hematopoiesis

Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Margarida Saraiva
da
Doutora Ana Cumano
e do
Doutor Jorge Pedrosa

março de 2018

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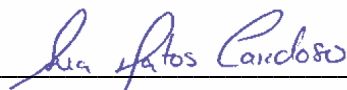
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List of Manuscripts

The work presented in this *Thesis* collects the following scientific article published and manuscript under preparation for submission in international peer-reviewed journals:

Ana Cardoso, A. Gil Castro, Ana Catarina Martins, Guilhermina M. Carriche, Valentine Murigneux, Isabel Castro, Ana Cumano, Paulo Vieira and Margarida Saraiva. *Frontiers in Immunology*, February 2018, DOI: 10.3389/fimmu.2018.00400. The dynamics of Interleukin-10-Afforded Protection during Dextran Sulfate Sodium-induced colitis. [*Thesis Chapter II*]

Ana Cardoso, A. Gil Castro, Isabel Castro, Ana Cumano, Paulo Vieira and Margarida Saraiva. The anti-inflammatory cytokine IL-10 is a new determinant of hematopoietic lineage commitment. *Manuscript under preparation*. [*Thesis Chapter III*]

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Abstract

Interleukin (IL)-10, an anti-inflammatory cytokine produced in all immune responses, stands out as a major inhibitor of inflammation. While the effect of pro-inflammatory cytokines in hematopoiesis is well recognized, little is known about the role that anti-inflammatory cytokines portray in the homeostasis maintenance of the hematopoietic system. The work described in this Doctoral Thesis aimed at expanding the current knowledge of how IL-10 impacts hematopoietic differentiation, and to what extent anti-inflammatory scenarios can modulate hematopoietic homeostasis, contributing to the development of hematologic disorders.

We described a novel mouse model of inducible IL-10 expression (pMT-10 mice) and investigated how an anti-inflammatory scenario, provided by transient IL-10 over-expression, could modulate cell maturation profiles in response to induced pathology. Our approach consisted in using a model of dextran sulfate sodium (DSS)-induced colitis, as IL-10 signaling, particularly in macrophages, is essential for intestinal homeostasis. pMT-10 mice pre-conditioned with IL-10 for 8 days before DSS administration showed a milder colitic phenotype. This protection was due to a reduction in the inflammatory profile of $Ly6C^+$ cell subset. Finally, we observed that IL-10 protection against DSS-induced colitis was not long-lasting. Further studies are required to fully elucidate the cellular and molecular bases of IL-10 short-term induced protection. However, this work highlights new possibilities for the mechanisms allowing IL-10 to control intestinal inflammation.

We next aimed at exploring a role for IL-10 in medullary hematopoiesis. It is well established that the cell fate decisions occurring during the hematopoietic process depend on external cues. Immunologic stress, as in the context of neoplasias and infection, changes the hematopoietic output to guarantee a constant supply of required immune cells. A role for IL-10 in hematopoiesis, although largely unknown, is expected, as several studies show the involvement of this cytokine in the pathogenesis of hematopoietic disorders. We found that IL-10 over-expression in pMT-10 mice led to several hematological alterations, namely i) increased myeloid cell production in the BM; ii) anemia; and iii) extramedullary myelopoiesis. The hematologic alterations observed required signaling through the IL-10 receptor (IL-10R) complex, since pMT-10 animals deficient in the IL-10Rα chain display a normal phenotype. Moreover, transplantation assays showed that both lymphoid and myeloid cells are key players in IL-10-driven myeloexpansion. We hope to wrap our findings by identifying the IL-10 target population and assessing the existence of other mediators involved in the phenotype.

Overall, this work reveals how IL-10 over-expression impacts hematopoiesis, and adds to our

understanding of the control of normal hematopoietic differentiation.

Resumo

A interleucina (IL)-10 é uma citocina anti-inflamatória produzida no decorrer de todas as respostas imunes, que se destaca como um importante inibidor de inflamação. Apesar de o efeito das citocinas pró-inflamatórias na hematopoiese estar bem descrito, pouco se sabe sobre o papel que as citocinas que inibem as reações inflamatórias apresentam na homeostase do sistema hematopoiético. O trabalho descrito nesta Tese de Doutoramento teve como objectivo enriquecer o conhecimento atual de como a IL-10 afeta a diferenciação hematopoiética e, em que medida cenários anti-inflamatórios podem regular a homeostase hematopoiética, levando ao desenvolvimento de doenças hematológicas.

Exploramos o modo como um cenário anti-inflamatório, proporcionado por uma sobre-expressão transitória de IL-10, pode modular os perfis de maturação celular em resposta a uma patologia induzida. Para este efeito, a nossa abordagem consistiu em usar um modelo de colite induzida por DSS, pois a sinalização de IL-10, particularmente em macrófagos, é essencial para a homeostase intestinal. Usando um modelo novo de sobre-expressão condicional de IL-10 (pMT-10), pré-condicionamos os ratinhos com IL-10 durante 8 dias, antes da induzirmos a doença. Os animais mostraram uma patologia menos acentuada. Esta proteção foi ainda mais notória aquando de um aumento da concentração de zinco no organismo dos animais. Esta observação deve-se possivelmente a uma redução no perfil inflamatório na população de células Ly6C$^+$. Por fim, observamos que a proteção conferida pela IL-10, contra a colite, não é persistente. Deste modo, são necessários mais estudos para esclarecer as mecanismos celulares e moleculares pelos quais a IL-10 confere uma proteção transitória contra colite. No entanto, este trabalho demonstra novas possibilidades para o mecanismo pelo qual a IL-10 controla a inflamação intestinal.

Exploramos depois um papel definitivo para a IL-10 na hematopoiese. As decisões que determinam o diferente tipo de diferenciação celular, que ocorrem durante o processo hematopoiético, dependem de sinais externos. O stress imunológico, por exemplo, durante cancro e infecção, altera a produção de células hematopoiéticas, de modo garantir um fornecimento adequado de células imunes. Uma função para a IL-10 na hematopoiese é esperada, dado que vários estudos mostram o envolvimento desta citocina no desenvolvimento de doenças hematopoiéticos. Neste trabalho descobrimos que a sobre-expressão de IL-10 em ratinhos pMT-10 leva a várias alterações hematológicas, como, i) aumento da produção de células mielóides na medula; ii) anemia; e iii) mielopoiese extramedular. As alterações hematológicas observadas requerem sinalização através do receptor da IL-10 (IL-10R), uma vez que os animais pMT-10, sem o IL-10R, apresentam um fenótipo normal. Além disso, experiencias de reconstituição do sistema imune demonstraram que tanto as

células linfóides como as mielóides estão envolvidas na expansão mielóide induzida pela IL-10. Esperamos fechar este mecanismo de expansão mielóide identificando a população-alvo da IL-10 e avaliando a existência de outros mediadores envolvidos no fenótipo.

Em suma, este trabalho demonstra como a sobre-expressão de IL-10 afeta a hematopoiese e enriquece o nosso conhecimento sobre o controle da diferenciação hematopoiética normal.

Palavras-chave: IL-10, hematopoiese, mielóide, diferenciação e células T

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CHAPTER II

The Dynamics of Interleukin-10-Afforded Protection during Dextran Sulfate Sodium-induced Colitis (Tables in Chapter II are part of the published manuscript)

List of Abbreviations

AGM	Aorta-gonad-mesonephros
AKT/PKB	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BCR	B-cell receptor
BM	Bone marrow
BrDU	Bromodeoxyuridine
Bupa	Buparlisib
C/EBP	CCAAT/enhancer-binding protein
cDC	conventional Dendritic cells
CFU	Colony forming units
CLP	Common lymphoid progenitors
CLR	C-type lectin receptors
CML	Chronic myelogenous leukemia
CMP	Common myeloid progenitors
DC	Dendritic cells
DN	Double negative
DP	Double positive
EBF	Early B cell factor
Epo	Erythropoietin
Ery/Mk	Erythroid/megakaryotic
ET	Essential thrombocythemia
FL	Fetal liver
FS	Fetal spleen
FT	Fetal thymus

G-CSF	Granulocyte colony stimulating factor
GATA-1	GATA-binding protein 1
GATA-2	GATA-binding protein 2
GATA-3	GATA-binding protein 3
GF1	Growth factor independence 1
GM	Granulocytic-macrophage
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocytic-macrophage Progenitors
GSK3	Glycogen synthase kinase 3
GWAS	Genome-wide association study
HE	Hematoxylin-eosin
HSC	Hematopoietic stem cells
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-10R	IL-10 Receptor
ILC	Innate lymphoid cells
IRF	Interferon regulatory factor
IV	Intravenously
JAK	Janus kinase
Lin	Lineage
LMPP	Lymphoid primed multipotent progenitors
LPS	Lipopolysaccharide
LSK	Lineage$^-$ Sca-1$^+$ c-Kit$^+$ cells
LT-HSC	Long-term hematopoietic stem cells
LTis	Lymphoid tissue inducer cells
MDS	Myelodyplastic syndrome

MEP	Megakaryocyte erythrocyte progenitors
MHC	Major histocompatibility complex
MPN	Myeloproliferative neoplasm
MPP	Multipotent Progenitor
MYD88	Myeloid differentiation primary response gene
Mφ	Macrophage
NETs	Neutrophil extracellular traps
NK	Natural killer
NKp	NK cell precursors
PB	Peripheral blood
PBMCs	Peripheral blood mononuclear cells
PI	Propidium iodide
PI3K	Phosphoinositide-3-kinase
PMF	Primary myelofibrosis
PU1.1	Purine rich box-1
PV	Polycythemia vera
RAG	Recombination activating gene
RBC	Red blood cells
ROR	RAR-related orphan receptor
ROS	reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
Ruxo	Ruxolitinib
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
ST-HSC	Short-term hematopoietic stem cells
STAT	Signal transducers and activators of transcription
SyK	Spleen tyrosine kinase
TCR	T cell receptor

TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll like receptors
TNF	tumor necrosis factor
Tpo	Thrombopoietin
Treg	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoietin
Tyk2	Tyrosine kinase 2
WHO	World health organization
YS	Yolk sac
Zn	Zinc
γc	Gamma chain

CHAPTER I

General Introduction

1.1 Hematopoiesis

Hematopoiesis refers to the process by which mature blood cells are produced [1-3]. During embryonic development, sequential independent waves of hematopoiesis have been described [4-7]. The mature blood cells produced in the first wave are referred to as primitive. Later, enucleated erythrocytes, macrophages, megakaryocytes and even some lymphoid cells appear, being the hematopoietic stem cells (HSCs) the last ones to be produced [4, 6]. Two major properties define these cells. Firstly, they possess the ability of self-renewal, being able to generate more HSCs. Second, they have the capacity to differentiate into all the progenitor subsets that give rise to all the different hematopoietic lineages [8, 9]. The end result of these events is the sustained and balanced production of cells of all lineages.

In the next sections two particular topics will be discussed. Following a brief introduction to the origin of HSCs, the mechanisms modulating hematopoietic commitment and the outcome when the organism faces the failure of these regulatory factors will be addressed.

1.1.1 Ontogeny of Hematopoietic Stem Cells

Hematopoiesis develops in primary hematopoietic organs. These differ between fetal and adult life and provide the signals necessary to sustain blood cell differentiation [9]. In vertebrates, definitive HSCs first emerge during embryonic development near the dorsal aorta-gonad-mesonephros (AGM) region [10, 11]. Analysis of the AGM niche has unveiled the support of a number of major signaling and physiological pathways, such as inflammation, in the generation of HSCs [12, 13]. Interestingly, it has been shown that the inflammatory response pathway is required for HSC emergence. This pathway is triggered by the toll-like receptor (TLR) 4 [12, 13], which is normally activated by bacterial infection. However, in the context of development, where there is no infection, its mechanism of activation remains unknown.

The first hematopoietic precursors are generated in the yolk sac (YS) around embryonic day 7 and give rise, mainly, to primitive erythrocytes and macrophages. The first steps of hematopoiesis are transitory, and are characterized by several consecutive waves with increased complexity [4-7, 9]. Their main function is to support the development of the growing embryo by supplying oxygen, tissue remodelling and conferring immunity [12]. Definitive HSCs emerge at embryonic day 8 in the paraaortic splanchnopleura that evolves to the AGM around embryonic day 9.5. Upon fetal liver (FL) colonization, at embryonic day 10, the HSC pool

rapidly expands and starts differentiation [9, 14, 15]. Fetal thymus (FT) also plays a role in the hematopoietic process, from its development to throughout life. Around embryonic day 11, T cell migration initiates FT colonization. As FT does not contain self-renewing progenitors, it requires a constant replenishment of progenitors to sustain thymopoiesis [16]. Fetal spleen (FS) has also a transient contribution to the early steps of hematopoiesis by supporting myeloid differentiation from embryonic day 13 until shortly after birth [17]. Finally, HSCs colonize the bone marrow (BM) around embryonic day 15, where they become resident cells. At the end of gestation, FL hematopoiesis decreases and the BM assumes the production of hematopoietic cells throughout life [9]. While HSCs expansion in the FL is accompanied by active precursor proliferation, the majority of HSCs in the BM becomes quiescent shortly after birth [18, 19].

1.1.2 The Hematopoietic System

Circulating blood cells are the key mediators of organism homeostasis. As mature blood cells, they have relatively short life spans, requiring a constant turnover in order to maintain normal physiological numbers. Hematopoiesis refers to the overall process of blood cell production, beginning in the embryo and continuing throughout life [3, 20]. The hematopoietic process is a complex and hierarchic set of differentiation steps, at the apex of which is a rare subpopulation of cells, the HSCs. These cells have unique renewing abilities and, as they mature, their progeny varies widely [3, 8, 20, 21].

Initial studies, using cell transplantation, established the definition of HSCs and allowed for their isolation and characterization. Thus, the ability of normal adult single cells to renew and contribute to blood cell formation is the definitive piece of evidence that HSCs exist and are maintained throughout life [9, 20, 22-24]. Recent lineage tracing studies, in mouse models, corroborate findings in transplanted recipients that certain intermediate progenitors have a vast but finite proliferative potential [25-27]. Hence, over time there is a decreasing correlation between the numbers of primitive and differentiated cell types present.

Hematopoiesis is a systematic process, in which pluripotent HSCs give rise to a large number of self-renewing committed precursors that differentiate into mature blood cells. This process is marked by a progressive loss of the differentiation potential, traceable by changes in cell surface markers [8, 28]. The identification of stem and progenitor cells by Weissman and collaborators led to the construction of a hematopoietic lineage tree that is characterized by a

cascade of binary decisions [29-31]. In the BM, different progenitors were described: the common lymphoid progenitors (CLPs) that give rise to all lymphoid cells [30]; the common myeloid progenitors (CMPs) that generate granulocytes and macrophages progenitors (GMPs) and the megakaryocyte and erythrocyte progenitors (MEPs) [29].

The lymphoid lineage is responsible for the adaptive immunity and provides a life-long immunity following exposure to pathogens. CLPs give rise to natural killer cells (NK), pro-B and pro-T cells, uncommitted lymphoid progenitors that will differentiate further into mature B and T cells (Figure 1.1) [30]. NKs are considered to be the bridge between the innate and adaptive immunity [32]. Aditionally, CLPs generate innate lymphoid cells (ILCs), that include the lymphoid tissue inducer cells (LTis), a subset of cells responsible for the generation of secondary lymphoid tissues during embryonic development [33, 34].

Curiously, functionally equivalent and phenotypically indistinguishable myeloid and lymphoid dendritic cells (DCs) can be derived from either CMPs or CLPs (Figure 1.1) [35, 36]. The myeloid linage comprises the first line of defense of the organism against external stimuli, being the foundation of innate immunity. GMPs give rise to macrophages (Mϕ), neutrophils, eosinophils and basophils/mast cells (Figure 1.1) [29, 37]. MEPs will originate erythrocytes and megakaryocytes that are respectively responsible for oxygen delivery and clotting [29]. The erythro-megakaryocytic lineage rapidly branches out, being currently accepted by many that this differentiation is the first branching point during hematopoiesis [38].
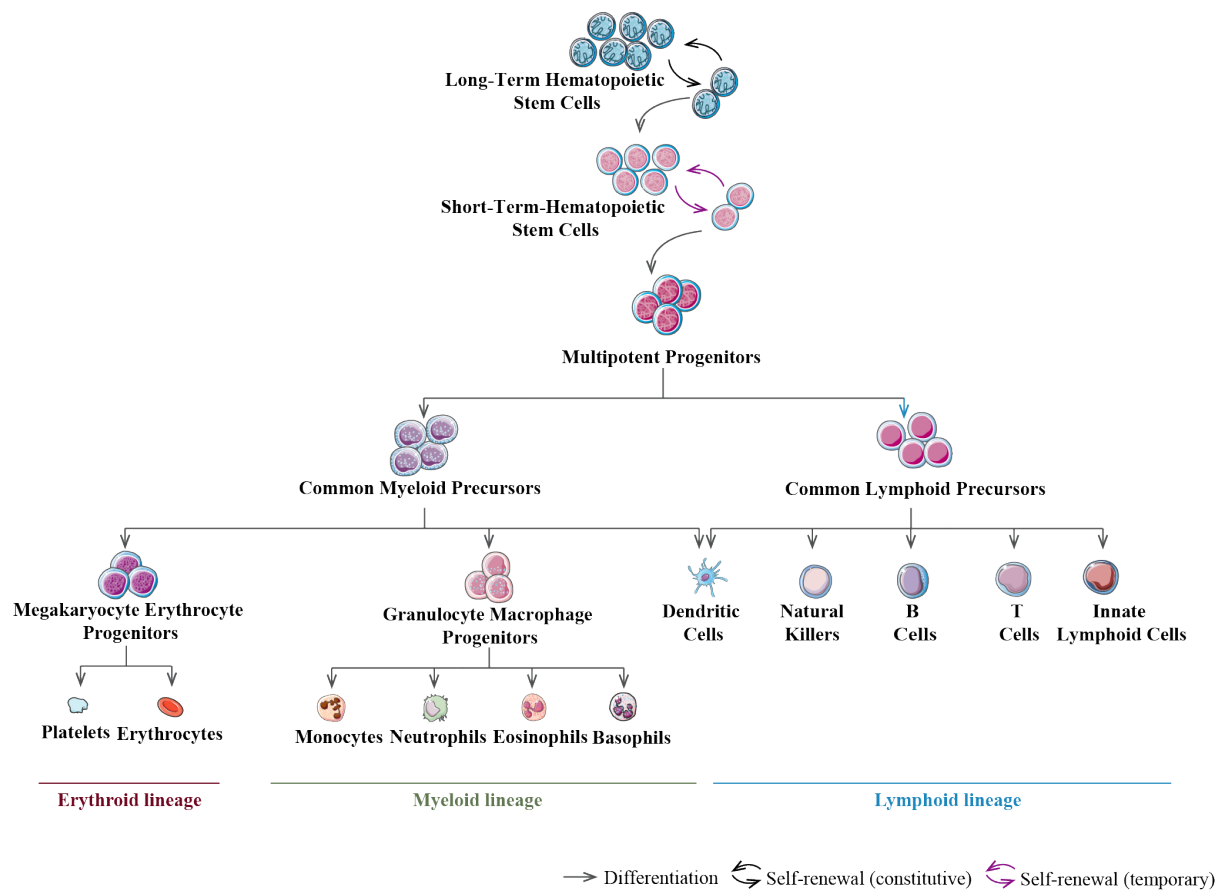
Fig1.1. Schematic of adult mouse hematopoiesis.

1.1.3 Lineage Restriction

HSCs are the first identified and best characterized adult stem cells. The hematopoietic process is conventionally described as a hierarchical system, where HSCs undergo lineage restriction as they are progressively driven down specific pathways into distinct cell types, losing their renewing properties until they terminally differentiate. The long-term (LT)-HSCs reside at the top of the hematopoietic pyramid [8, 20]. The LT-HSCs first differentiate into short-term (ST)-HSCs, which comprise the multipotent progenitors (MPPs). MPPs can be found, in the mouse model, in a fraction of BM cells that lack the expression of differentiated cell surface markers and express high levels of c-Kit (CD117) and Sca-1 [8, 31, 39]. They are referred to as lineage negative (Lin) Sca-1$^+$ c-Kit$^+$ cells (LSKs).

In the LSK pool, LT-HSCs and ST-HSCs can be distinguished by the expression of additional markers as Thy, Flt3 and CD34 (Thy1^{lo} Flt3$^-$ CD34$^-$ and Thy1^{lo} Flt3$^-$ CD34$^+$ respectively) [22, 40]. LT-HSCs can be further discriminated by using the CD150 and CD48 markers (CD150$^+$ CD48$^-$) [41]. Importantly, Flt3 expression within LSKs marks the loss of self-renewal ability and identifies MPPs with transient multilineage reconstitution ability (LSK Flt3$^+$)

[42, 43]. At this stage, a crossroad for the different lineage pathways appears, as MPPs give rise either to CLPs (Lin- Interleukin (IL)-7Rα+ Thy1- c-Kit^{lo} Sca-1^{lo}) or CMPs (Lin- IL-7Rα- c-Kit^{lo} Sca-1^{lo} FcγR^{lo} CD34+) [29, 30]. Subsequently, CMPs give rise to MEPs (Lin- IL-7Rα- c-Kit^{lo} Sca-1^{lo} FcγR- CD34^{lo}) and GMPs (Lin- IL-7Rα- c-Kit^{lo} Sca-1^{lo} FcγR+ CD34^{hi}) [29]. The identification of CLPs and CMPs led to the hypothesis that the first commitment step occurring at the MPP stage is a binary event, where cells become committed either to the myeloid/erythroid or lymphoid lineages. However, several groups have questioned this model as they identified a lymphoid-myeloid progenitor with no erythrocyte/megakaryocytic potential. Adolfsson and colleagues showed that the Flt3^{hi} fraction of MPPs contains a lymphoid MPP with lymphoid and myeloid potential but no erythroid/megakaryotic (Ery/Mk) potential [44]. Additional reports demonstrated the heterogeneity of the MPP compartment using VCAM-1 and Flt3 [45, 46]. They showed that VCAM-1+ Flt3+ LSKs can reconstitute the granulocytic-macrophage (GM) and lymphoid compartment, while VCAM-1- Flt3^{hi} LSKs have a residual Ery/Mk and GM potential, giving rise mainly to lymphoid cells. Thus, they were able to define that the high expression of Fl3 in the LSK compartment marks the loss of Ery/Mk potential and expression of lymphoid transcripts, defining the lymphoid-primed MPPs (LMPPs). Single cell analysis also showed that lymphoid and Ery/Mk transcriptional programs are exclusive to MPPs, thus suggesting that the Ery/Mk potential is lost before lymphoid priming in LMPPs [47].

Hematopoiesis current model acknowledges that HSCs self-renewal ability is associated with multipotency and that HSCs lose differentiation potential in a stepwise manner in the process of leukocytes maturation. According to this model, hierarchical differentiation proceeds with the first lineage commitment at the MPP stage, giving rise to all mature blood lineages. Despite wide acceptance of this concept, evidence from which hierarchical associations were built are sometimes doubtful. Recently, several groups questioned the current scheme of lineage commitment mostly because LMPPs with both GM and lymphoid potentials, but little or no Ery/Mk potential, have been detected, suggesting an alternative Ery/Mk differentiation pathway. These observations led to a revised model of hematopoiesis where the first event in HSC differentiation is the loss of their self-renewal capability and progression to the MPP stage [44, 45, 48]. However, the issue of a branching point of Ery/Mk lineage remains controversial. Interestingly, recent studies suggest a model in which there is no linear lineage restriction where commitment occurs gradually, by self-regulation of lineage-associated programs in intermediate progenitors [47, 49].

The establishment of all hematopoietic lineages during development is tightly controlled by transcription factors that act in a sequential and parallel fashion, building lineage-specific networks and circuits. Lineage commitment can be induced by several factors, as external cues, cytokines, direct cell-cell interactions or transcription factors [8, 20]. Recently, microRNAs have also been shown to play a regulatory role in lineage commitment [50, 51]. Both extrinsic and intrinsic factors may have an instructive role and actively induce commitment and differentiation or simply be tolerant for the growth of pre-committed progenitors by promoting cell survival and/or expansion.

1.1.3.1 Lymphoid Commitment

Lymphoid commitment is marked by the upregulation of the IL-7Rα [52]. The characterization of the CLP subset was difficult to achieve, as the cues leading to maturation of the lymphoid subsets are unique to the different mature populations. The establishment of a functional immune system with diverse antigen receptors is dependent on the V(D)J recombination activating gene (Rag)1 and Rag2 products. These two proteins constitute the key lymphoid components required for the activation of antigen receptor rearrangement. Thus, functional disruption of either Rag1 or Rag2 genes leads to immunodeficiency due to lymphoid arrest at a stage prior to the recombination of the antigen receptor loci. In Rag-deficient mice, both T and B-cell differentiation is impaired due to the absence of antigen receptors.

T lymphocytes differentiate in the thymus from progenitors that migrate from the FL, during embryogenesis, and the BM, after birth and throughout life [9, 53]. Thymopoiesis is a well-described process where antigen receptor genes rearrangement and determination mechanisms of self-tolerance ensure a well-balance development. T cell differentiation, on one hand, requires activation of the Notch signaling pathway and repression of transcription factors, vital to B cell development, as Pax5 [54, 55]. Considering the different developmental perspectives discussed in the previous section, it becomes evident that the process of B-lymphocyte commitment involves two basic features, one being the loss of all other lineage potentials and the second being a gain of characteristics crucial for differentiation of the B-lineage pathway.

Hardy and colleagues established one of the major schemes of B cell development, based on the combined expression of B220, BP-1, CD24 and CD43, and by their

Immunoglobulin (Ig) gene rearrangement status [56]. Later on, CD19 expression was described as a key marker of commitment to the B cell lineage, allowing discrimination of B cell precursor populations [57]. A fully rearranged B cell receptor (BCR) defines immature B cells [58]. Cells are then evaluated for self-tolerance and migrate to the spleen were they maturate. The molecular mechanism surrounding B cell development involves a series of transcription factors, such as Purine rich box-1 (PU.1), Ikaros, early B cell factor (EBF), E2A and Pax5 [52, 54]. Inactivation of any of these factors induces a severe phenotype by impairing B cell differentiation at an early stage [59-63]. Importantly, Pax5-deficient pro-B cells can also originate macrophages, DCs and granulocytes in response to macrophage (M)- colony stimulating factor (CSF), granulocyte-macrophage (GM)-CSF and granulocyte (G)-CSF, respectively [64].

ILCs are typically classified into three distinct groups based on their production of T helper (h) cell-associated cytokines [33]. Thus, group 1 comprises interferon (IFN)-γ-producing ILCs, including NK cells and ILC1 cells. Group 2 comprises IL-5 and IL-13-producing ILCs, which are dependent on GATA-binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor-α (RORα) for their development. Group 3 comprises IL-17 and/or IL-22-producing ILCs that are dependent on the transcription factor RORγt for their development [33]. ILC development is dependent on the common cytokine receptor γ-chain (γc). While ILCs are dependent on IL-7 signaling, NK cell development requires IL-15 signaling [34, 65].

Despite the fact that NK cells mainly differentiate in the BM, several reports describe the presence of immature forms of NKs in extra-medullary sites as the thymus suggesting that NK development can occur, in some rare cases, in different organs [66, 67]. NK progenitors (NKp) have high NK cell potential but lack B, T, erythroid and myeloid potential. NKp give rise to immature NKs that start to express NKp46 and NK1.1 [68-70]. Maturation of NK cells occurs through the upregulation of DX5 and CD43 proteins [68, 71]. Similarly to T cells, NKp pass through a selection process were they acquire self-tolerance. Mature and competent NK cells, with cytotoxic activity, leave the BM and colonize different organs as spleen, lungs and gut.

Several reports suggest that not only transcription factors have regulatory roles in hematopoiesis, but also cytokine receptor signaling. The involvement of cytokines in hematopoiesis will be addressed in section 1.1.4.

1.1.3.2 Myeloid Commitment

The most abundant populations of mature immune cells are of myeloid nature. The myeloid compartment includes circulating monocytes, tissue specific Mϕ, DCs, granulocytes (neutrophils, eosinophils and basophils) and mast cells, being all of them progeny of GMPs [72, 73]. The distinguishable feature of terminal subsets of the myelopoietic branch is their ability to broadly recognize invading pathogens and endogenous danger signals. This key surveillance role leads to the rapid engagement of key mechanisms that facilitate the elimination of infectious agents and the restoration of homeostasis.

Monocytes circulate in the bloodstream and enter tissues, mainly under emergency conditions, where they differentiate into tissue Mϕ. However, they provide only a limited contribution to the tissue-resident populations of Mϕ that are seeded early in embryogenesis [72, 73]. Neutrophils and monocytes are circulating cells that enter the tissues under homeostatic conditions, where they are phagocytized and eliminated by resident Mϕ [74]. Under emergency conditions, they display essential effector antimicrobial activities that range from the production of large amounts of reactive oxygen species (ROS), to the decondensation and consequent release of chromatin to generate the neutrophil extracellular traps (NETs) [75].

Conventional DCs (cDCs) differ from other myeloid cells due to their capacity for efficient antigen presentation and their ability to secrete high levels of cytokines [73]. Eosinophils are cytotoxic effector cells with antimicrobial and antiviral activity [76]. Basophils and mast cells, generally associated with type 2 inflammatory responses, are key effector cells for the clearance of parasitic infections. However, they can also play a role in allergic responses [77]. Although most circulating granulocytes are short-lived cells, with a lifespan of only a few hours or days, mast cells, which share some basic features with basophils, never circulate in the peripheral blood as mature cells and can survive in their tissue of residence four weeks or months [78].

The machinery governing lineage commitment is undoubtedly very complex. The regulation of gene expression is not a linear process, but it is instead the result of a multifarious interplay between chromatin modifications, transcription factors and signaling molecules [50]. Interestingly, it has been found that the cell fate and identity within the myeloid lineage is controlled by a limited number of key transcription factors [50]. Levels of these molecules are therefore tightly regulated within the hematopoietic tree, hinting that any alteration in their expression may impact the outcome of mature cells drastically. One of such

transcription factors is PU.1 that controls HSCs maintenance as well as the production of CLPs and CMPs, as stated before [79-81]. Low expression of PU.1 favors B cell differentiation while high concentration of this factor promotes macrophage differentiation [82]. Disruption of GATA-2, a transcription activator mostly expressed at early progenitor level, perturbs HSCs niche, leading to a monocytopenia, mild neutropenia, and DCs, B and NK deficiency [83-86]. Within the myeloid branch, the CCAAT/enhancer-binding protein alpha (C/EBPα) is also a key transcription factor for GM commitment. Conditional inactivation of C/EBPα leads to reduced numbers of CMPs and block their differentiation into GMPs, affecting the numbers of all downstream lineages [87]. While, C/EBPα-deficient mice do not express the G-CSF receptor (R), exhibiting a blockade in early granulocyte development, C/EBPε expression is required only at the final steps of granulocyte maturation [8]. Similarly to C/EBPε, absence of the growth factor independence (GFI) 1, known to preserve HSC functional integrity [88], blocks the later steps of granulocyte development [89]. Another important transcription factor is IFN regulatory factor 8 (IRF-8), as its deficiency causes an increased granulocytic output and the defective production of monocytes, mast cells and basophils [90]. An important role was also attributed to GATA-1 regarding erythropoiesis, as its deficiency in embryos, blocks the development of erythroid cells, leading to a lethal anemia. GATA-1 was also shown to be essential to the maturation of Mk [91].

Other molecular players involved in lineage commitment are the miRNAs. Several miRNAs, such as miR-233, miR146 and miR155, were shown to affect myeloid cell differentiation and function [50]. Overall, the main message is that differentiation of the distinct cell subsets within the myeloid lineage involves several complementary factors that are not exclusive to a single cell type.

While the aforementioned factors are absolutely essential for the survival and proliferation of progenitor cells, other molecules, such as cytokines, strongly impact the cell fate decisions of stem cells, both for symmetric expansion and lineage commitment.

1.1.4 Modulation of Hematopoiesis by Cytokines

Most breakthroughs regarding lineage commitment were derived from technologies allowing for cell isolation and combination of different cell surface markers and, genetic strategies, by gene targeting deletion and over-expression experiments. Numerous groups, over the past years,

have uncovered a great deal of information regarding the mechanisms by which cytokines modulate hematopoiesis [8, 92, 93]. Several transcription factors are induced by the presence of cytokines in the microenvironment where lymphopoiesis and myelopoiesis are occurring.

IL-7 plays an essential role in B cell differentiation, due to the fact that this cytokine is uniquely able to maintain the expression of EBF in BM derived CLPs and to promote the expansion and survival of pro-B cells [94]. Other studies demonstrated that IL-7 also impacts T lymphopoiesis, since in the presence of mutations in *IL7* or *IL7R* genes result in a dramatic block of T cell development in the thymus [94-98]. Interestingly, another 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 [99]. Like IL-7, IL-21 has also been shown to play a key role in B cell development. 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 [100-102]. Other cytokines, such as type I IFN, are known to strongly inhibit proliferation of pro-B cells and consequently B cell development [103]. IFNα in particular, is able to activate dormant HSCs, by promoting their efficient exit of G0 phase and entrance in the active cell cycle, thus stimulating the proliferation of HSCs. However, IFNαR-mediated signaling is not required for the HSCs proliferation and differentiation [104].

Two experimental approaches were developed to quantify myeloid progenitors, that allow for an assessment of progenitor frequency and their proliferative and differentiation potential [105]. The first one is based in quantifications of progeny cells, through *in vitro* colony-forming unit (CFU) assays; and the second one is based in phenotypic characterization, using monoclonal antibodies. Clonal assays for GMPs demonstrated the essential requirement for M-CSF, GM-CSF and IL-3 for the generation of macrophages *in vitro* [106]. Interestingly, this does not seem to be the case *in vivo* as M-CSF/ GM-CSF or IL-3-deficient mice, develop normally, presenting only a slightly lower number of GMs than their wild type counterparts [107, 108]. Moreover, mice deficient for all M/G/GM-CSF still present all GM populations, despite a pronounced phenotype, characterized by a high susceptibility to bacterial pneumonic when challenged with thioglycolate [109]. GM-CSF also presents an important role in cell differentiation since its deficiency leads to impaired pulmonary homeostasis and increased splenic hematopoietic progenitors, with unimpaired steady-state hematopoiesis [110]. Nonetheless, they still present all GM populations. Additional evidence for the role of G-CSF

signaling in myelopoiesis came through a study reporting that mice lacking G-CSFR exhibit a chronic neutropenia and decreased numbers of progenitors [111].

IL-3 was also shown to act synergistically with other cytokines, such as IL-6 and G-CSF on committed and uncommitted myeloid progenitors [92, 112]. IL-6 is able to support emergency granulopoiesis in animals that lack G-CSF and GM-CSF, two critical cytokines necessary for myelopoiesis [113, 114]. Also, *in vivo* studies demonstrated that IL-12 plays an important role in hematopoiesis, by suppressing cell differentiation in the BM, enhancing (splenic) extramedullary hematopoiesis and mobilizing hematopoietic progenitor cells to the peripheral circulation [115, 116]. The ensemble of these studies shows a partial, and often synergistic, effect of these cytokines in myelopoiesis, suggesting the involvement of additional factors in this process.

In addition to lymphopoiesis and myelopoiesis, the generation of erythrocytes is also regulated by cytokines such as IL-3, that has been shown to positively regulate early erythropoiesis, causing rapid cell proliferation and increased cell survival of myeloid progenitors [117-120].

Altogether, the role of cytokines, mainly pro-inflammatory ones, in hematopoiesis has been addressed and their contribution to the hematopoietic decision is well established. An important piece of information to have in mind is the fact that cytokine expression varies during life, either because of external cues, as infection, or by intrinsic changes as homeostatic imbalances. All of these fluctuations impact hematopoiesis homeostasis leading, in extremes cases, to the development of hematologic pathologies.

1.1.5 Deregulation of Hematopoiesis

As aforementioned, hematopoiesis is a highly regulated process that consists on a strict balance between self-renewal and differentiation of HSCs along lymphoid or myeloid lineages. Among the hematopoietic modulators growth factors as ILs, IFNs and transcription factors have been shown to play key roles in cell differentiation and maturation, by delivering critical cues to HSCs and progenitors. Interestingly, a connection between several of these molecules, their receptors and hematopoietic malignancies was also shown, demonstrating that alterations in their expression are at the base of many hematopoietic disorders such as leukemias, lymphopenias and myeloproliferative neoplasms (MPNs) [121-124].

MPNs comprise a group of hematologic disorders characterized by an HSC-derived expansion of at least one myeloid subset [125]. These conditions develop primarily in the BM, but depending on the disorder subtype can impact the liver and/or the spleen [125]. According to World Health Organization (WHO) guidelines, myeloid neoplasms are classified as chronic neutrophilic leukemia, chronic eosonophilic leukemia, chronic myelogenous leukemia, mastocytosis, essential thrombocytopenia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) [125]. These last three disorders are subcategorized as Philadelphia-chromosome-negative classic MPNs [126-128]. PMF in particular is caused by an expansion of HSCs in the BM [127, 128]. In this pathology cell proliferation is accompanied by a reactive nonclonal fibroblastic proliferation and fibrosis of the BM. 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 [126-128]. As the BM becomes fibrotic and normal hematopoiesis can no longer happen, extramedullary hematopoiesis starts taking place – leading to a splenomegaly and liver enlargement [129].

The most commonly recognized mutation in classic MPNs is the JAK2 V617F mutation [128]. This mutation is found in more than 90% of patients with PV and in approximately 50% of those with ET or PMF and is responsible for constitutive activation of the JAK/ signal transducer and activator of transcription (STAT) signaling pathway [128]. These percentages reinforce the idea that other genetic factors and even signaling pathways are involved in the development of MPNs. In PV patients lacking this mutation, a similar activating JAK2 exon 12 mutation can be found [128]. Approximately 10% of patients with PMF and ET 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]. Other genes, such as IDH1/2, ASXL1, DNMT3A and CalR, were found to contribute to epigenetic regulation of JAK2 [128, 130, 131]. Mutations in ten-eleven-translocation (TET2), required for DNA hydroxymethylation, affect mostly HSCs and progenitors and are found in approximately 14% of MPNs [128, 132]. Also, mutations in RUNX1 have been associated with transformation of MPNs to acute myeloid leukemia, contributing to the severity of the hematological disorder [128].

The importance of JAK-STAT pathway in MPNs is demonstrated by the wide availability of target therapies, classified as single-agent JAK inhibitors or combination of drugs with JAK-inhibitors [124, 133, 134]. For example, ruxolitinib (Ruxo) is a JAK inhibitor Food and Drug

Administration and European Medicines Agency approved, and most prescribed drug to treat MPNs, being associated with symptom improvement and reduction of risk of death [135]. 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 [136]. In addition to JAK-STAT signaling, other molecular pathways are under study as therapeutic targets for MPNs [137, 138]. Aberrant phosphoinositide 3-kinase (PI3K) signaling has already been extensively associated with several types of neoplasms, due to its role on cell proliferation and survival [139, 140]. Unveiling the association of PI3K with hematologic conditions allowed the discovery of several genetic mutations associated with the increased risk of disease. In MPNs, BM samples from patients showed an increased phosphorylation of AKT, which is indicative of the activation of the PI3K pathway [141]. Owing to the numerous reports on PI3K aberrant activation in hematologic conditions, a large number of inhibitors have been developed, as is the case of Buparlisib (Bupa)[133].

Despite the importance of these discoveries and their applications, much remains to be elucidated as for the mechanisms that link imbalances in hematopoietic modulators to disease initiation and maintenance, and how this knowledge can be translated into specific, mechanism-based therapies.

1.2 IL-10

Mosmann and collaborators firstly described IL-10 as "cytokine synthesis inhibitory factor" (CSIF), a product of Th2 cells, that inhibited IFN-γ production by Th1 cells [142]. Subsequently they characterized cDNA clones encoding mouse and human IL-10 that were found to share a sequence homology with a gene in the Epstein-Barr virus genome [143, 144]. Later on, IL-10 was shown to have other remarkable immunomodulatory properties by impairing cytokine, chemokine and nitric oxide production from macrophages upon bacterial lipopolysaccharide (LPS) stimulation [145]. In 1993, Kuhn and colleagues made an important discovery in the field, establishing a correlation between IL-10 expression, T cells, myeloid cells and intestinal homeostasis [146]. The group demonstrated that IL-10 is a critical molecule for the balance of intestinal homeostasis, by showing that IL-10 deficient mice spontaneously develop inflammatory bowel disease (IBD) promoted by the animal facility conditions. This was subsequently supported by the observation that IL-10R-deficient mice also develop spontaneous colitis under the same conditions [147]. These findings uncovered IL-10 role in mediating immune cell functions, and led to an extensive research effort aiming to elucidate the role of IL-10-dependent signaling in the regulation of intestinal immune function [148, 149]. Until today IL-10 remains one of the most studied and enigmatic regulatory molecules, with several different functions attributed to this cytokine.

1.2.1 IL-10 in Health and Disease

The immune response has evolved to protect the host from a wide range of potentially pathogenic microorganisms. Nonetheless, if unregulated, the same mechanisms have the potential to cause damage to the host. IL-10 has a unique role in the negative regulation of inflammatory responses, primarily through selectively limiting the expression of genes encoding pro-inflammatory cytokines, chemokines, and cell-surface molecules [145, 149]. However, IL-10 expression can also inadvertently hamper immune responses, contributing to chronic infection. Therefore, IL-10 is an important immunoregulatory cytokine and understanding the mechanism underlying IL-10 regulation is crucial for the development of new therapeutics.

IL-10 has been shown to play an important role in the pathogenesis of numerous disorders, such as IBD, Systemic lupus erythematosus (SLE), rheumatoid arthritis, psoriasis, multiple sclerosis and certain lymphomas, through its immunosuppressive properties [148,

150-155]. As previously mentioned, IL-10 was shown to have an important role in IBD as its deficiency leads to pathology [148-150]. IL-10 conferred protection in various models of colitis such as T cell transfer induced colitis [156], DSS-induced colitis [157, 158] and in colitis induced by streptococcal peptidoglycan-polysaccharide polymers [159]. Also, IL-10 production is required for recovery, since patients that respond to steroids have high IL-10 levels during and after treatment [160]. The treatment of IL-10 deficient mice only fully protects from colitis if administered before the disease is established [161]. Taking into consideration the results obtained in murine models of IL-10 perturbation and the anti-inflammatory properties of IL-10, this cytokine presented as a very promising candidate for the treatment of IBD. In fact, there is now proof that similarly to the clinical cases of IL-10 and IL-10R loss of function mutations, steroid therapy does not work in patients that have low levels of IL-10. However, in the clinical trials performed so far, IL-10 offered modest effects of protection only [162]. More recently, genome wide association studies (GWAS) further demonstrated that IL-10 is associated with the development of IBD [150, 163, 164].

IL-10 has also been studied in the context of other autoimmune diseases. Numerous groups have demonstrated the complexity of IL-10 axis in the pathogenesis of murine lupus [165-168]. However, conflicting findings have been reported too as to whether IL-10 may play a disease promoting or protective role in lupus-prone mice models. In most lupus animal models, elevated IL-10 production has been described. Interestingly, IL-10 polymorphisms T-3575A, G-2849A and C-2763 (that enhance *Il10* expression) are associated with an increased risk in patients with SLE [169]. In a clinical trial, anti-IL-10 administration to lupus patients with cutaneous and joint manifestations resulted in improvement to clinically inactive disease in five of six patients within 6 months of the 3-week treatment regimen [170]. A better understanding of the regulation of IL-10 and signaling pathway may likely provide more valuable information to the pathogenic mechanisms underlying specific forms of SLE, so as to pave the way toward more effective therapeutics.

Finally, IL-10 also regulates immune responses against pathogens [171]. In the context of infection, excess of IL-10 is often associated with chronicity as in the case of *Streptococcus pneumoniae* and *Klebsiella pneumoniae* [172, 173], whereas its absence can lead to an excessive uncontrolled pro-inflammatory response during infection with *Toxoplasma gondii*, inducing exacerbated host tissue damage [174].

These studies, altogether, highlight that IL-10 function may result in distinct outcomes during different pathologies, ranging from the control of otherwise protective T-cell responses, mediating the pathway to chronic infection, to fatal host-mediated pathology. Thus, IL-10 manages a delicate balance between suppressing and activating host responses to wide range of pathogens.

1.2.2 IL-10-mediated effects in the immune response

Induction of the IL-10-mediated anti-inflammatory responses occurs through the binding to its surface heterodimeric membrane receptor, the IL-10R, a transmembrane protein formed by 2 subunits, a α and β chain [145, 175-177]. IL-10Rα is expressed in most leukocytes and serves as the ligand binding subunit. IL-10Rβ is constitutively expressed in most cell types and serves as the signaling subunit [145]. Most hematopoietic cells express the IL-10Rα at a basal level. Nonetheless it can be upregulated upon activation, highlighting IL-10 importance in the modulation of multiple steps of the innate and adaptive immunity. Huber and colleagues have shown that *in vivo* anti-CD3 treatment induces IL-10Rα expression on Th17 cells in the small intestine [178]. In addition, *in vitro* stimulation of mouse naïve T cells with anti-CD3, effector T cells and regulatory T cells (Tregs) leads to upregulation of IL-10Rα expression [179]. Similarly, at steady state, human neutrophils express basal levels of IL-10Rα. However, following LPS or IL-4 stimulation, IL-10Rα expression is upregulated [180]. Moreover, Corinti and colleagues have shown that human DCs become unresponsive to IL-10 after maturation by downregulating IL-10Rα surface expression, enabling them to produce higher levels of pro-inflammatory mediators and to prime T cells [181]. Unlike IL-10Rα, which is unique to IL-10, the IL-10Rβ subunit is shared by receptor for other type-II cytokines including IL-22R, IL-26R, IL-29R and IFNγ [145].

1.2.2.1 IL-10 signaling through the IL-10R

The interaction between the protein IL-10 and its receptor leads to the activation of transduction pathways, via the JAK/STAT complex [182] (Figure 1.2). Upon IL-10 binding, the interaction between IL-10/ IL-10Rα leads to conformational changes required for the engagement with IL-10Rβ to be possible [183]. Once the IL-10/ IL-10Rα/ IL-10Rβ complex is assembled, tyrosine kinases JAK1 and tyrosine kinase (Tyk) 2 are recruited and associated

with IL-10Rα and IL-10Rβ, respectively [175, 177, 184]. The tyrosine kinases become active and promote the phosphorylation of the IL-10Rα on two tyrosine residues, leading to the recruitment of STAT. Following its recruitment, phosphorylation of STAT3 by Jak1 and Tyk2 occurs, leading to its homodimerization and subsequent translocation to the nucleus, where it binds to STAT3-binding elements of IL-10-responseive genes [184, 185]. STAT3 also induces the expression of STAT3-dependent suppressor of cytokines (SOCS3), which binds to PRR-induced expression of various inflammatory cytokines including TNF, IL-6 and IL-1β [184]. Interestingly, both IL-10 and IL-6 highly induce SOCS3 expression in Mϕ with different outcomes, as the inhibitory role of SOCS3 appears to be restricted to IL-6 [186, 187]. While SOCS3 may play a role in driving-specific outputs, the details underlying those mechanisms remain unknown.

STAT3 is essential for all known functions of IL-10, as this molecule mediates gene expression that executes IL-10-mediated anti-inflammatory responses [186]. Nonetheless, the involvement of other proteins of the STAT family has been shown in the context of IL-10 signaling. Interestingly, IL-10R activation via JAK2 has also been reported in the context of *in vitro* stimulation of lymphoma cells from patients with diffuse large B-cell lymphoma [188]. Moreover, a different signaling pathway for IL-10 responsiveness was unveiled recently: the PI3K/ AKT/ Glycogen synthase kinase (GSK) 3 pathway [189] (Figure 1.2). This pathway is associated with several cellular functions, including proliferation, differentiation, metabolism, growth and survival [190]. Several groups have studied the role of PI3K signaling in response to IL-10 [189, 191, 192]. Their findings on human Mϕ stimulated with LPS have shown that PI3K is able to increase IL-10-mediated inhibition of induced IL-1, IL-8 and cyclo-oxygenase-2 [189]. Moreover, Gunzl and colleagues, who used a model of constitutive activation of PI3K on Mϕ, observed that cells adopted a strong anti-inflammatory profile via up-regulation of IL-10 [191]. These studies reinforce the relevance of PI3K signaling pathway on the response to IL-10 and IL-10 action.
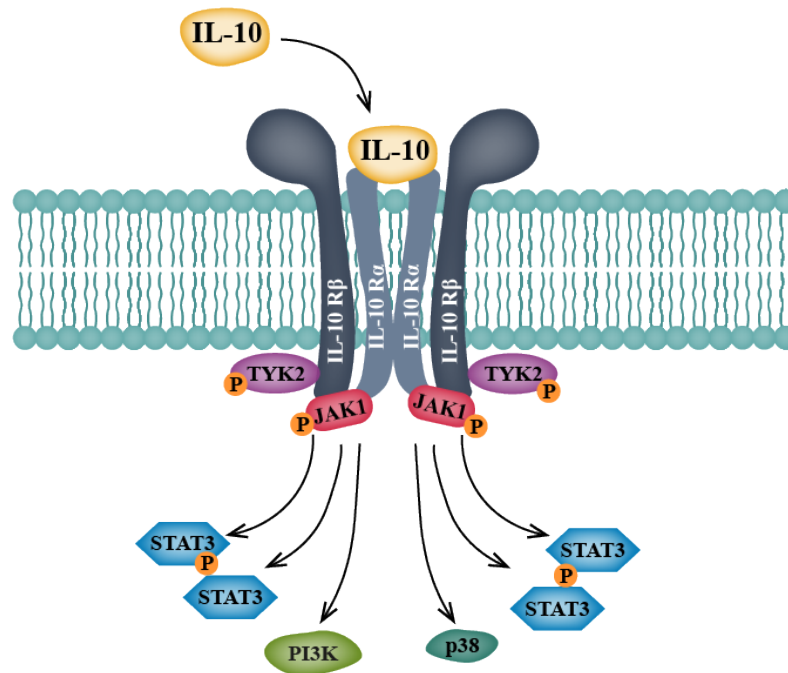
Fig 1.2. The IL-10/IL-10R signaling pathway.

1.2.2.3 IL-10 target cells and molecules

IL-10 is a key anti-inflammatory cytokine produced and recognized by a wide range of leukocytes, as well as non-hematopoietic cells. IL-10-producing cells include populations from both innate and adaptive immunity. Among innate immune cells, Mϕ and classic DCs are the main producers of IL-10, although eosinophils, neutrophils and mast cells are also able to produce this cytokine in response to a variety of stimulus. Among adaptive immune cells, B cells and different subsets of T cells, such as Th1, Th2 and Th17 cells, have been shown to secrete IL-10 [145, 193, 194].

Triggering of the IL-10R, by IL-10, dampens the cytokine and chemokine production, and expression of co-stimulatory molecules, such as CD80, CD86, and major histocompatibility complex (MHC) Class II, and of its receptors in monocytes/ Mϕ and DCs [195-199]. It also increases nitric oxide production in Mϕ [200]. Due to the autocrine inhibitory effect of IL-10 on Mϕ and DCs, IL-10 inhibits the development of Th1 type responses but also leads to the suppression of Th2 cells and allergic responses [201]. Therefore, an additional feedback loop exists to limit the innate effector functions of Mϕ and DCs and their subsequent activation of T cells [201, 202]. However, IL-10 enhances the differentiation of IL-10-secreting Treg cells, thus providing a positive regulatory loop for its induction [203]. In some situations,

IL-10 also activates mast cells and enhances the functions of CD8^{+} T cells, NK cells and B cells [145, 204-207]. So, IL-10 is a cytokine with important effector properties that impacts the development of an immune response.

1.2.3 IL-10 in hematopoiesis

Placing IL-10 as a modulator of cell differentiation and maturation can be expected as most hematopoietic cells express the IL-10R, thus being potential targets of IL-10. In fact, since its discovery, several groups have shown evidence that IL-10 may play a role in hematopoiesis. Kang and colleagues reported that HSCs stimulation with IL-10 enhances their self-renewal potential [208]. Through the use an IL-10 deficient mouse model and *ex vivo* cultures, the authors observed a decrease of progenitor hematopoietic populations in the BM of IL-10 deficient mice; an enhanced HSC regeneration in stromal cultures that produce IL-10; and, induction of HSC self-renewal in purified LSK cultures stimulated with exogenous IL-10. Based on these observations, the authors hypothesized that IL-10 may constitute a bridge between the immune and hematopoietic systems. Another *ex vivo* study showed a pronounced expansion of myeloid progenitor cells when CD34^{+} cells, cultured with a standard cocktail for cell survival, were supplemented with recombinant IL-10 [209]. Oehler and colleagues studied the effect of IL-10 on spontaneous hematopoietic colony formation in normal human peripheral blood mononuclear cells (PBMCs) [210]. Their findings place IL-10 as a regulator of hematopoiesis by mediating the release of GM-CSF by accessory cells. They concluded that IL-10 suppresses spontaneous myeloid colony formation by PBMCs, and its suppressive potential is restored upon the addition of GM-CSF. In all, results from the different studies seem to be contradictory, but one has to consider that the different isolation techniques used, as well as the different culture conditions, may account for the differences observed. Other reports evaluating the role of cytokines in hematopoiesis have positioned IL-10 as an indirect modulator of this process. Vlasselaer and co-workers showed, that IL- 10 was able to stimulate the production of GM colony forming units by blocking transforming growth factor (TGF)-β synthesis by the osteogenic stroma, promoting hematopoiesis in this microenvironment [211, 212]. Thompson-Snipes and colleagues showed that IL-10, synergistically with IL-3 and IL-4, modulates cell proliferation by its growth-promoting activity on mast cells, megakaryocytes and multilineage colonies derived from committed progenitors and Thy1^{low} SCA-1^{+} [204]. In another study, by Rennick and colleagues, the differentiation potential of IL-10 was measured by

colony-forming assays, having the authors concluded that IL-10 alone does not lead to the generation of any mature cells, yet, when in combination with other cytokines, such as IL-3, IL-6, Epo or IL-11, promotes the growth of megakaryocyte colonies [213]. There are also evidence, *in vitro,* indicating that IL-10 acts synergistically with Epo significantly increasing erythroid differentiation and proliferation [214].

An additional piece of evidence arose, though indirectly, from the results of IL-10 clinical trials. IL-10 administration was reported to cause anemia and splenomegaly, characteristic features of myelopoiesis-associated pathologies.

Interestingly, a bidirectional effect of IL-10 on early B cell development has been reported. By stimulating Flt3 with IL-7, IL-10 can induce pro-B cell formation and growth before differentiation into a more mature form, characterized by CD19 expression. On the other hand, IL-10 has been suggested to inhibit the growth of pro-B cells after differentiation, *i.e.,* after they start to express CD19 [215].

Overall, these reports were unclear in providing a specific role for IL-10 in hematopoietic differentiation. 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 vague and awaits clarification.

1.3 Context and Aims of this Thesis

Cell fate decisions within the hematopoietic process result from interactions between the BM microenvironment and external cues. Immunologic stress, such as cancer and infection, changes the magnitude and composition of the hematopoietic output to guarantee the supply of immune cells to the increased demand. IL-10, produced during all immune responses, stands out as a major inhibitor of inflammation. Interestingly, while the effect of pro-inflammatory cytokines in hematopoiesis is well described, little is known of the role that cytokines that inhibit inflammatory reactions, such as IL-10, play in the homeostasis of the hematopoietic system.

To contribute for a better understanding of how IL-10 impacts hematopoietic development and to which extent anti-inflammatory scenarios can modulate hematopoietic homeostasis, adding or aiding to hematologic diseases, I took advantage of a genetically modified mouse line (pMT-10) previously generated, in our lab, to over-express IL-10. Thus, in the present thesis, I sought to:

To characterize a novel mouse model of inducible IL-10 expression and probe it in the experimental colitis model. [**Thesis Chapter II**]

To determine in detail the effect of IL-10 expression in the differentiation of the different hematopoietic cell lineages and the molecular mechanisms underlying it. [**Thesis Chapter III**]

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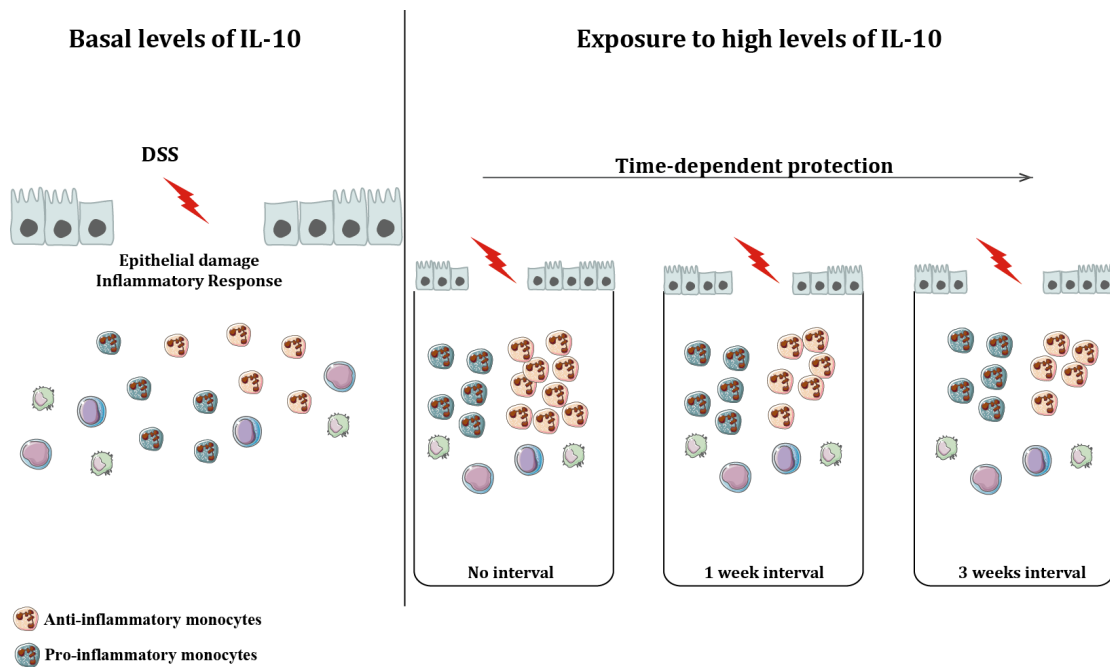
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The Dynamics of Interleukin-10-Affored Protection during Dextran Sulfate Sodium-induced Colitis

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Graphical Abstract

The Dynamics of Interleukin-10-Afforded Protection during Dextran Sulfate Sodium-Induced Colitis

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

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

INTRODUCTION

Inflammatory bowel disease (IBD) comprises a complex group of inflammatory conditions of the gastrointestinal tract (1) affecting an increasing number of patients worldwide (2–4). Both forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC), result from alterations in the immune homeostasis of the intestinal tissue leading to local uncontrolled inflammation (5, 6). The gut is a very particular site in terms of immune repertoire and regulation, as even in homeostatic conditions constant exposure to antigens occurs (7). Thus, the maintenance of intestinal homeostasis, primarily

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carried out by intestinal macrophages, requires a constant and fine-tuned balance between the state of tolerance and inflammation (8). In the gut environment, macrophages encounter a plethora of stimuli, from dietary antigens to commensal bacteria, yet, due to their unique tissue-specific characteristics, remain tolerant (9). In the predisease stage, the epithelial or mucosal barriers become compromised allowing bacteria from the luminal side to invade the *lamina propria* of the gut (10). This event triggers an acute inflammatory response due to the activation of immune cells by direct contact with bacterial products (10). The induced inflammation results either in elimination of the foreign bacterial incursion or in an exacerbated immune response that can result in tissue damage. The damage caused by deregulated inflammation will perpetuate the activation of effector cells and ultimately lead to the clinical onset of IBD (10, 11).

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

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

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

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

MATERIALS AND METHODS

Ethics Statement

In Portugal, all animal experiments were performed in strict accordance with recommendations of the European Union Directive 2010/63/EU and previously approved by Portuguese National Authority for Animal Health–Direção Geral de Alimentação e Veterinária (DGAV). Mice were euthanized by CO_2 inhalation with efforts to minimize suffering.

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

Animals

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

Generation of pMT-10 Mice

pMT10 mice were generated by A. Gil Castro and Paulo Vieira. Mouse IL-10 cDNA was cloned into the p169ZT vector, which

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carries a sheep metalloprotein (MT) 1a promoter, a β-globin splice site and a SV40 polyadenylation (polyA) signal. The resulting vector—pMT-10 (see **Figure 1A**)—was then injected into C57BL/6j eggs and transgenic founders were identified by PCR using MT and IL-10-specific primers. IL-10 overexpression was induced by feeding the mice a 2% sucrose solution with 50 mM of zinc (Zn) sulfate.

DSS-Induced Colitis

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

Assessment of Intestinal Inflammation

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

criteria scores were added to give an overall inflammation score of 0–12.

Cytokine Quantification

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

Preparation of Cell Suspensions

Isolation of non-hematopoietic cells (CD45⁻ TER119⁻) or hematopoietic (CD45⁺) BM cells followed standard protocols. Briefly, hematopoietic BM cells were extracted by flushing the femurs and tibias with 2 mL of HBBS repeatedly. To obtain the BM non-hematopoietic cells, the bone fragments were incubated in RPMI medium with Liberase TL (0.5 mg/ml; Roche) for 30 min at 37°C. To help dissociation of non-hematopoietic cells from the bone, after each incubation period, the femurs and tibias were flushed with RPMI. We repeated this step three times after which we flushed the bones one last time, harvested the cell suspensions and added 1 volume of RPMI containing 10% FCS. Small intestine (SI), non-hematopoietic cells were isolated as previously described (42). Skin non-hematopoietic cells were isolated from ear samples. Samples were harvested and the epidermis exposed by separating the external layers. Epidermis was incubated for 45 min at 37°C with Liberase HL (0.5 mg/ml; Roche) and DNase I (1 U/mL; Invitrogen). After 45 min, non-hematopoietic cells were dissociated from the tissue by mechanical disruption, collected, washed in HBSS containing 10% FCS and recovered. At the end, non-hematopoietic cells were sort-purified by excluding all
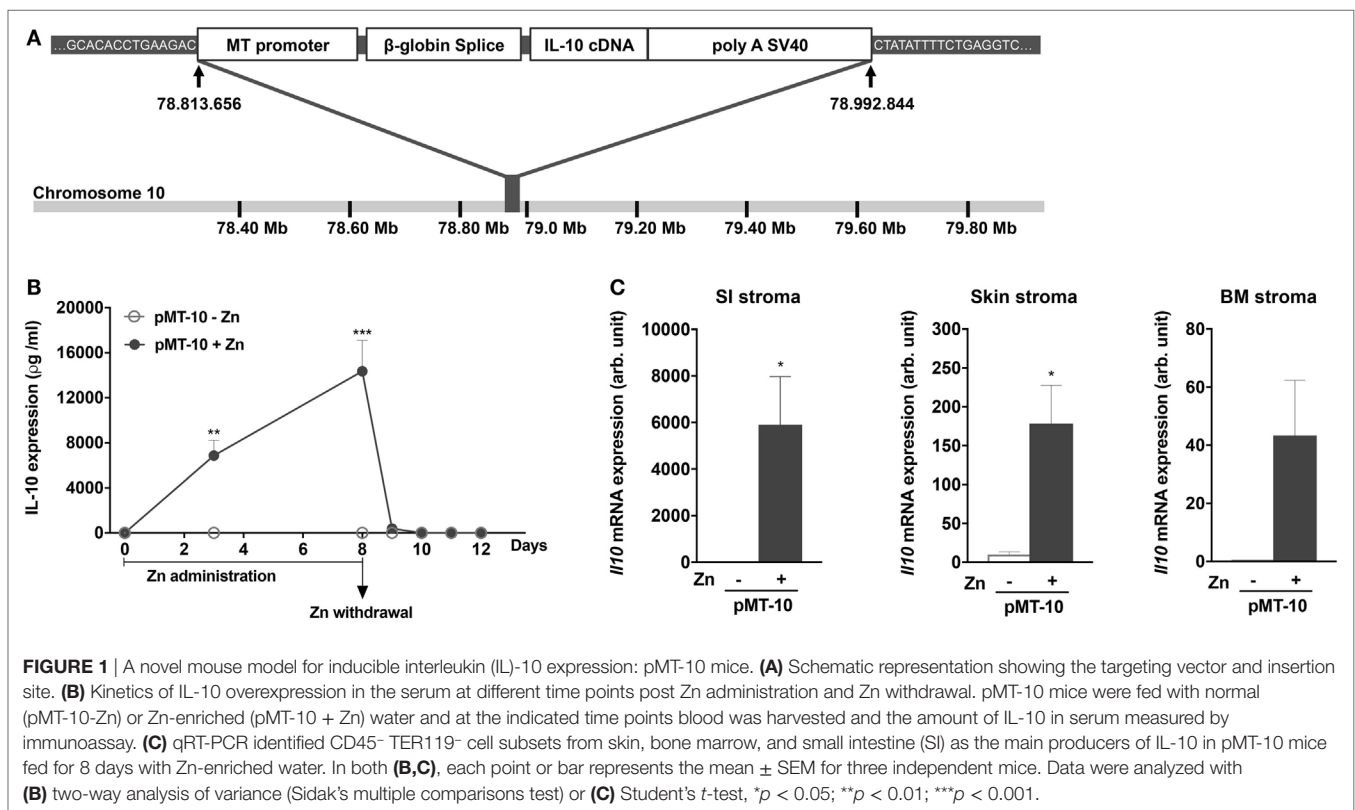

FIGURE 1 | A novel mouse model for inducible interleukin (IL)-10 expression: pMT-10 mice. **(A)** Schematic representation showing the targeting vector and insertion site. **(B)** Kinetics of IL-10 overexpression in the serum at different time points post Zn administration and Zn withdrawal. pMT-10 mice were fed with normal (pMT-10-Zn) or Zn-enriched (pMT-10 + Zn) water and at the indicated time points blood was harvested and the amount of IL-10 in serum measured by immunoassay. **(C)** qRT-PCR identified CD45⁻ TER119⁻ cell subsets from skin, bone marrow, and small intestine (SI) as the main producers of IL-10 in pMT-10 mice fed for 8 days with Zn-enriched water. In both **(B,C)**, each point or bar represents the mean ± SEM for three independent mice. Data were analyzed with **(B)** two-way analysis of variance (Sidak's multiple comparisons test) or **(C)** Student's *t*-test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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TABLE 1 | Disease Activity Index (DAI) parameters.

Score	Weight loss	Stool consistency	Bleeding
0	No loss	Normal	No blood
1	1–5%	Mild soft	Brown color
2	5–10%	Very soft	Reddish color
3	10–20%	Diarrhea	Bloody stool
4	>20%		Gross bleeding

DAI is obtained by the sum of each individual score.

TABLE 2 | Parameters for histological analysis of colitis severity.

Score	Epithelial hyperplasia and goblet depletion	Leukocyte infiltration in the *Lamina Propria*	Area affected	Markers of severe inflammation
0	None	None/rare	None	None
1	Minimal	Increase	1/3	Minimal
2	Mild	Confluent	2/3	Increased
3	Marked	Transmural	All	Confluent

The final score is obtained by the sum of individual scores. Markers of severe inflammation included ulceration and crypt abscesses.

CD45^{+} TER119^{+} cells (Figure S1A in Supplementary Material). Cell suspensions for all other organs were obtained by mechanical disruption.

Lamina propria leukocytes (LPLs) were prepared as previously described (43). Briefly, LPLs were harvested, dissociated and resuspended in Hank's Balanced Solution (HBSS) supplemented with 1% fetal calf serum (FCS; Gibco). To isolate LPLs, the colon was flushed with phosphate-buffered saline (PBS; Gibco), opened and cut into 1 cm pieces. To eliminate epithelial cells these fragments were incubated at 37°C in Ca- and Mg-free PBS containing 10% FCS and 5.0 mM EDTA under strong agitation for 30 min. For LPL isolation, the remaining fragments were incubated in RPMI medium with Liberase TL (0.5 mg/ml; Roche) for 30 min at 37°C. To complete the digestion, the suspension was repeatedly passed through a 10 ml syringe for 5 min, filtered through a 40 μm cell strainer (BD Bioscience) and collected by centrifugation. The cell pellet was resuspended in 44% Percoll (GE Healthcare), laid over 67% Percoll, and centrifuged at 600 *g* for 20 min at 20°C. Cells at the interface were collected, washed in HBSS containing 1% FCS and recovered.

Antibodies
Antibodies were conjugated to fluorochromes (FITC, PE, PECy7, APC, APCCy7, Pacific Blue, and BV711) and were specific for the following mouse antigens: CD3 (145-2C11; Biolegend), CD11b (M1/70; Sony), CD11c (HL3; Biolegend), CD19 (6D5; Sony), CD45.2 (104; Biolegend), Ly6C (Hk1.4; eBioscience), Ly6G (RB6-8C5; BD Pharmingen), CD45 (30F11; Sony), and TER119 (Ter119; BD Pharmingen).

Cell Sorting and Multiplex Real Time-PCR Analysis
CD45^{−} TER119^{−} or CD45^{+} cells were sort-purified based on the expression of CD45 and TER119 using an Aria sorter (BD). Dead cells were eliminated by exclusion with propidium iodide (PI). mRNA from sorted cells was extracted using RNeasy Micro kit (Qiagen) and converted into cDNA by reverse transcription with PrimeScript RT Reagent kit (Takara, Clonetech). qRT-PCR was performed using Taqman primers (see Table S1 in Supplementary Material for references) and Taqman Universal Master Mix (Applied Biosystems). qRT-PCR reactions were performed on a ABI 7300 thermocycler (Applied Biosystems).

Lamina propria leukocytes were FACS-sort purified based on the expression of CD45.2, CD11b and Ly6C, using an Aria III sorter (BD). Cells expressing CD3, CD19, CD11c, and Ly6G were excluded. Dead cells were eliminated by exclusion with PI. CD45.2^{+} CD11b^{+} Ly6C^{+} cells were sorted directly into a mix of 9 μl of CellsDirect One-Step qRT-PCR kit (Life Technologies), containing a mixture of diluted primers (0.05× final concentration, see Table S1 in Supplementary Material for references). Preamplified cDNA (18 cycles), was obtained according to the manufacturer's instructions and was diluted 1:5 in TE buffer (pH = 8; Ambion). The sample mixture was as follows: diluted cDNA (2.9 μl), Sample Loading Reagent (0.32 μl; Fluidigm), and Taqman Universal PCR Master Mix (3.5 μl; Applied Biosystems). The assay mixture was as follow: Assay Loafing Reagent (Fluidigm) and Taqman Mix. A 48 × 48 Dynamic Array integrated fluidic circuit (IFC; Fluidigm) was primed with control line fluid, and the chip was loaded with assays and samples with and X IFC Controller (Fluidigm). The experiments were run on a BioMark HD (Fluidigm) for 40 cycles. Gene expression was normalized for *Hprt* and assessed by the $2\Delta^{Ct}$ method.

Statistical Analysis
Statistical analysis was performed with the Student's *t*-test or two-way analysis of variance as indicated in the figure legends. The analysis was performed with Prism Software (GraphPad). Graphs containing errors bars show means ± SEM. Statistical significance is represented as follows: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Generation of a Novel Mouse Model of IL-10 Overexpression
To study the biological impact of IL-10 overexpression in different settings, we engineered a novel mouse model to allow for inducible IL-10 expression, the pMT-10 mouse (44). For this, a construct containing the IL-10 cDNA under the control of the inducible sheep MT promoter was introduced in the genome of wild-type BL/6 mice (**Figure 1A**). Whole genome sequencing revealed a single insertion of the transgene in chromosome 10, between positions 78.813.656 and 78.992844 bp (**Figure 1A**). We estimated, by qRT-PCR, the number of copies of the transgene to be 50–100 (data not shown). The MT promoter is activated in the presence of 50 mM of Zn in the organism, administered in the drinking water. Kinetic analysis of IL-10 in the serum of pMT-10 mice fed with Zn-enriched water showed a rapid increase of circulating IL-10 (**Figure 1B**). Indeed, as soon as day 3 after IL-10 induction, the levels of this cytokine in the serum were very high (7–12 ng/ml)

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(**Figure 1B**). Moreover, suspending Zn administration led to a sharp drop in IL-10 in sera in only 24 h, to below detection levels in only 48 h (**Figure 1B**). As expected, circulating IL-10 was undetectable in pMT-10 mice fed with normal water (**Figure 1B**). Transcriptional analysis of different organs and cellular compartments of induced pMT-10 mice revealed that the expression of the exogenous IL-10 cDNA was restricted to CD45⁻TER119⁻ cells from the SI, skin and, to a less extent, BM (**Figure 1C**). IL-10 induction was not detected in the other organs analyzed (liver, spleen, kidney, choroid plexus, lung, and colon) nor in hematopoietic cells isolated from the BM (Figure S1B in Supplementary Material). Thus, the pMT-10 mouse model allows for timely controlled IL-10 overexpression in specific anatomic locations, accompanied by a strong increase of the levels of this cytokine in the serum.

DSS-Induced Colitis Is Ameliorated in IL-10 Preexposed Mice

Despite the clear link between low levels of IL-10 and susceptibility to colitis in human (45) as well as in mouse models (33, 34), administration of IL-10 to treat this condition showed only limited effects (37). A possible reason may be the poor accessibility of IL-10 to the site of inflammation. In this context, and in view of the high expression seen in the SI of induced mice, the pMT-10 mouse model offers an opportunity to further address the effects of IL-10 expression in the gut in the context of colitis. For this, we used the DSS experimental model, a highly reliable and reproducible way of causing UC-like symptoms in the mouse model by inducing acute inflammation with the recruitment of inflammatory cells (46).

We investigated the impact of IL-10 overexpression prior to DSS-induced colitis. For this, pMT-10 mice were induced to overexpress IL-10 for 8 days, before initiation of DSS administration (**Figure 2A**). As controls, non-induced pMT-10 or BL6 mice fed with control or Zn-enriched water were used. In our experimental setting, wild-type BL/6 mice started to show signs of disease from days 4 to 5 after administration of 3% DSS in the drinking water (**Figure 2B**). Control pMT-10 mice showed a progression of the DAI very similar to BL/6 mice (**Figure 2B**). As compared to pMT-10 or BL6 fed with control water, mice preconditioned with IL-10 showed significantly lower DAI after day 5 (**Figure 2B**) indicating that IL-10 conferred partial protection. Zn administration to BL6 mice prior to DSS, resulted in partial protection. Indeed, by day 7 of DSS administration, a significantly lower DAI was observed in BL/6 mice fed with Zn-enriched water as compared to BL6 control (**Figure 2B**). Nevertheless, the maximal protection was observed for pMT-10 mice overexpressing IL-10, which suggests a synergistic effect of IL-10 and Zn in the amelioration of the disease (**Figure 2B**). The DAI encompasses three scores, one of which is the weight loss. Relatively to control animals, both pMT-10 and BL/6 mice fed with Zn prior to DSS administration showed less weight loss (Figure S2 in Supplementary Material). pMT-10 mice preexposed to IL-10 showed the least reduction in colon length as compared to all control groups (pMT-10 or BL/6 fed with control water, and BL/6 fed with Zn-enriched water – **Figure 2C**), in line with
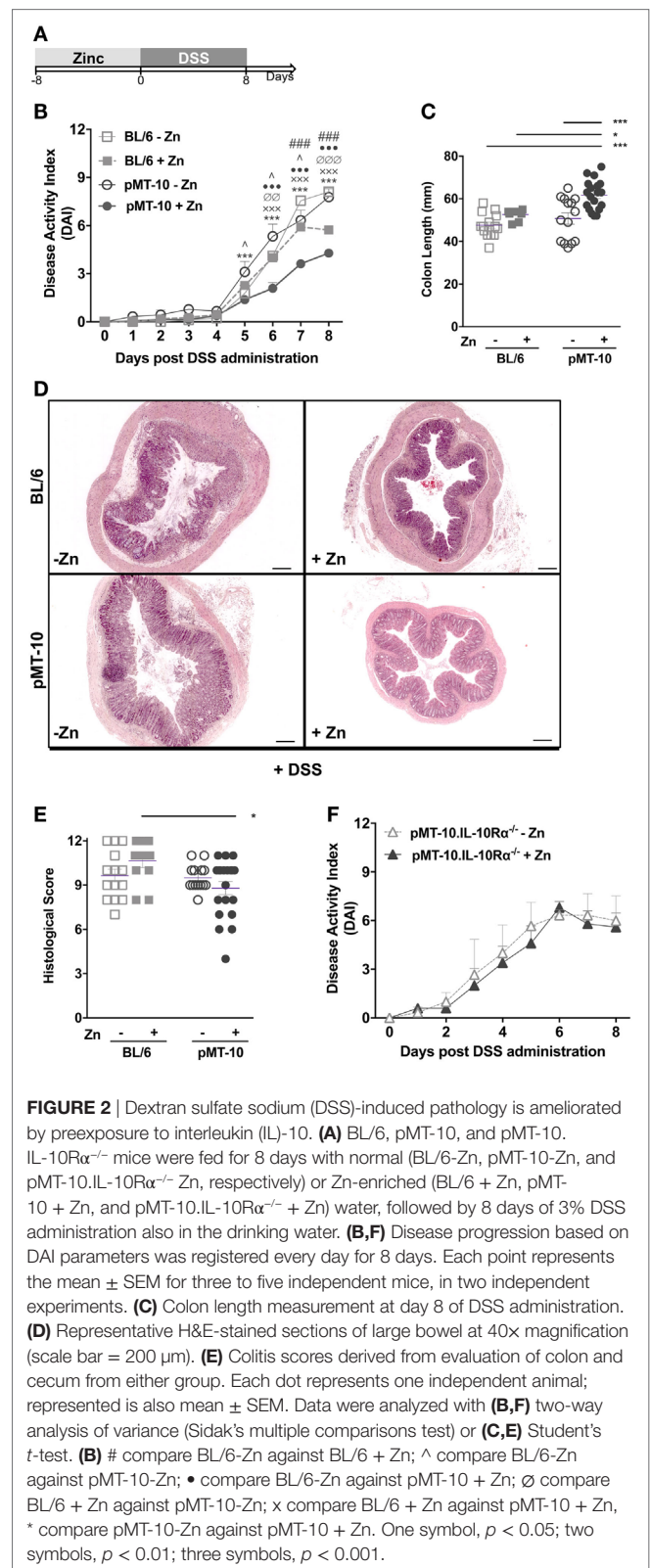

FIGURE 2 | Dextran sulfate sodium (DSS)-induced pathology is ameliorated by preexposure to interleukin (IL)-10. **(A)** BL/6, pMT-10, and pMT-10. IL-10Rα⁻/⁻ mice were fed for 8 days with normal (BL/6-Zn, pMT-10-Zn, and pMT-10.IL-10Rα⁻/⁻ Zn, respectively) or Zn-enriched (BL/6 + Zn, pMT-10 + Zn, and pMT-10.IL-10Rα⁻/⁻ + Zn) water, followed by 8 days of 3% DSS administration also in the drinking water. **(B,F)** Disease progression based on DAI parameters was registered every day for 8 days. Each point represents the mean ± SEM for three to five independent mice, in two independent experiments. **(C)** Colon length measurement at day 8 of DSS administration. **(D)** Representative H&E-stained sections of large bowel at 40× magnification (scale bar = 200 μm). **(E)** Colitis scores derived from evaluation of colon and cecum from either group. Each dot represents one independent animal; represented is also mean ± SEM. Data were analyzed with **(B,F)** two-way analysis of variance (Sidak's multiple comparisons test) or **(C,E)** Student's *t*-test. **(B)** # compare BL/6-Zn against BL/6 + Zn; ^ compare BL/6-Zn against pMT-10-Zn; • compare BL/6-Zn against pMT-10 + Zn; ∅ compare BL/6 + Zn against pMT-10-Zn; x compare BL/6 + Zn against pMT-10 + Zn, * compare pMT-10-Zn against pMT-10 + Zn. One symbol, *p* < 0.05; two symbols, *p* < 0.01; three symbols, *p* < 0.001.
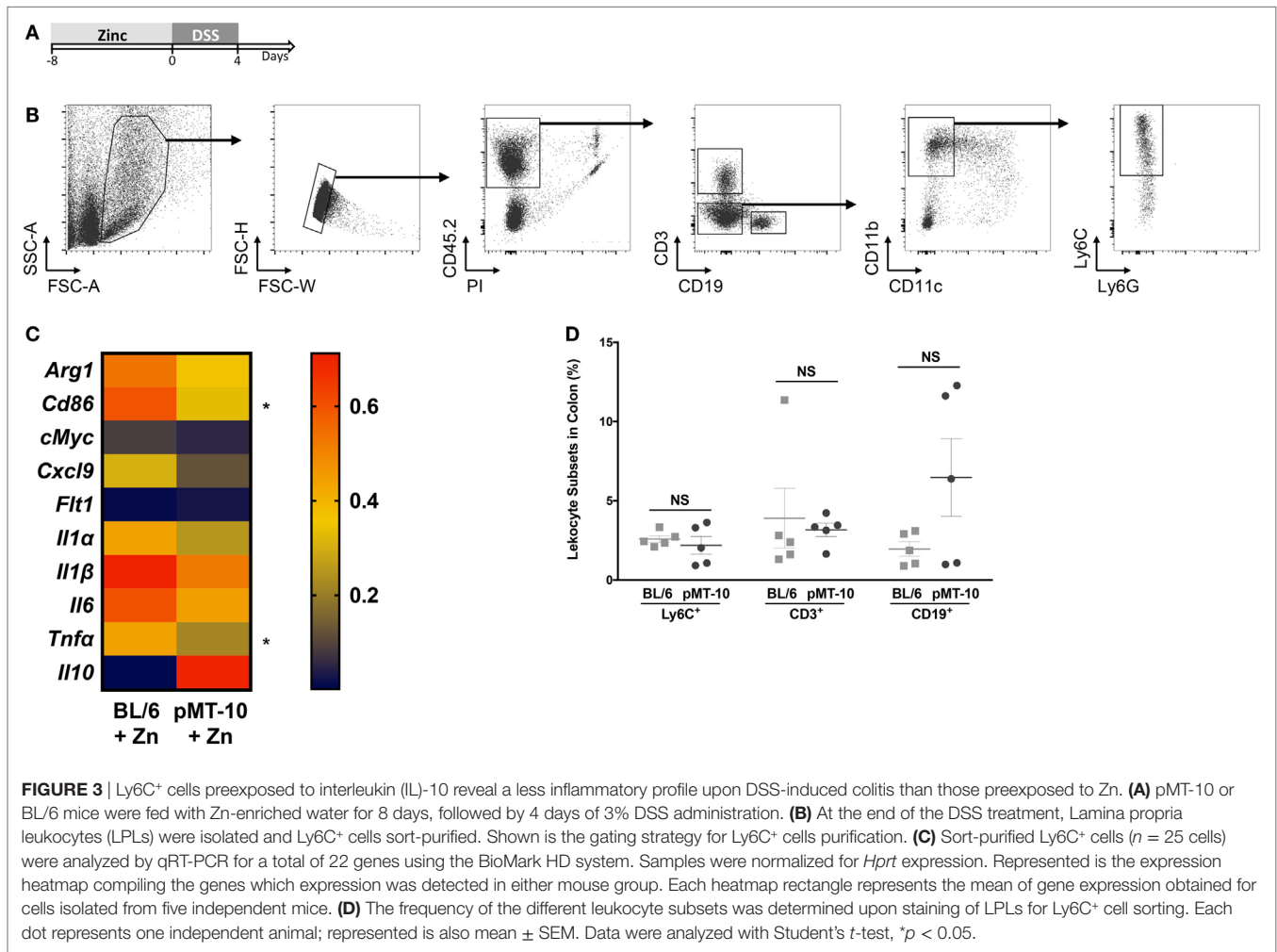
maximal protection being conferred by IL-10. Histologic analysis of the organ, comprising the analysis of inflammatory infiltrates, architectural distortion (crypt shortening and branching) and

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ulceration, showed an improvement in pMT-10 mice preexposed to IL-10 as compared to BL/6 preexposed to Zn (**Figures 2D,E**). However, despite a reduction in the histological score of some pMT-10 mice preexposed to IL-10, the overall group did not reach statistical significance when compared to the other control groups (**Figures 2D,E**).

Thus, our data showed that IL-10 overexpression prior to intestinal insult afforded a significant degree of protection from DSS-induced colitis. Additionally, our data also suggest a synergistic effect of IL-10 and Zn in the amelioration of the disease. To investigate this issue, we repeated the experiment using pMT-10 mice crossed with IL-10Rα$^{-/-}$ mice. Since pMT-10.IL-10Rα$^{-/-}$ double mutant mice are unresponsive to IL-10, the effects observed would only be due to Zn administration. In these mice, we observed an accelerated disease progression upon DSS administration, with an elevated DAI score as early as day 3, in line with the known role of IL-10 in controlling the disease (**Figure 2F**). In these mice, Zn administration did not confer protection against DSS-induced colitis (**Figure 2F**). Taken together, our findings support the notion that the protection conferred by Zn requires IL-10 signaling.

Preexposure to IL-10 Promotes a More Controlled Inflammatory Response

Previous studies have shown that monocytes and macrophages are the major effector subsets of colonic inflammation (34, 47). Mice with macrophage-specific IL-10R deficiency develop a spontaneous colitic profile, emphasizing the importance of IL-10 in regulating the macrophage response to prevent uncontrolled inflammation (34). Thus, we next investigated whether IL-10 ameliorated DSS-induced colitis by restricting the monocyte/macrophage response. Considering that Zn administration also improved the outcome of DSS-induced colitis in BL/6 mice, we compared the transcriptional profile of monocytes/macrophages from BL/6 or pMT-10 mice preexposed to Zn and subjected to DSS administration for 4 days. We chose this time point, since signs of colitis induced by DSS in both BL/6 and pMT-10 mice only become obvious after day 4 of DSS administration. Thus, BL/6 and pMT-10 mice were fed with Zn-enriched water for 8 days and then received DSS for 4 days (**Figure 3A**). At this time point, Ly6C$^+$ cells from each mouse from the different groups were FACS purified (**Figure 3B**). Expression of 22 genes (Table S1 in Supplementary Material) associated with the uncontrolled

FIGURE 3 | Ly6C$^+$ cells preexposed to interleukin (IL)-10 reveal a less inflammatory profile upon DSS-induced colitis than those preexposed to Zn. **(A)** pMT-10 or BL/6 mice were fed with Zn-enriched water for 8 days, followed by 4 days of 3% DSS administration. **(B)** At the end of the DSS treatment, Lamina propria leukocytes (LPLs) were isolated and Ly6C$^+$ cells sort-purified. Shown is the gating strategy for Ly6C$^+$ cells purification. **(C)** Sort-purified Ly6C$^+$ cells (n = 25 cells) were analyzed by qRT-PCR for a total of 22 genes using the BioMark HD system. Samples were normalized for *Hprt* expression. Represented is the expression heatmap compiling the genes which expression was detected in either mouse group. Each heatmap rectangle represents the mean of gene expression obtained for cells isolated from five independent mice. **(D)** The frequency of the different leukocyte subsets was determined upon staining of LPLs for Ly6C$^+$ cell sorting. Each dot represents one independent animal; represented is also mean ± SEM. Data were analyzed with Student's *t*-test, *p < 0.05.

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immune response developed in IBD were analyzed by multiplex RT-PCR. All samples, from both groups, expressed three house-keeping genes (*Hprt, Actb,* and *Gapdh*). Of the 22 genes analyzed, we failed to detect expression of 9 (*Il4, Il9, Il12α, Il12β, Il13, Il17, Il23, Ifnγ,* and *Cx3cl1*) in Ly6C+ cells isolated from the *lamina propria* in both groups. We detected expression of the 10 remaining genes in Ly6C+ cells, in Zn-fed BL/6 and pMT-10 mice after DSS administration (**Figure 3C**), but no expression in the absence of insult (data not shown). Thus, Ly6C+ cells alter their expression profile in response to DSS insult. Most interestingly, on day 4 post-DSS administration, Ly6C+ cells isolated from induced pMT-10 mice presented an overall less inflammatory profile than those isolated from BL/6 mice (**Figure 3C**). In the case of *Tnfα* and *Cd86*, the differences observed between the two mouse groups were statistically significant (**Figure 3C**). In all, these findings suggest that exposure to IL-10 before DSS induction acts by preventing an inflammatory profile in Ly6C+ cells.

Of note, the frequency of inflammatory macrophages recruited to the inflamed gut was similar between the two groups, and the same was true for CD3 T cells and CD19 B cells, showing that IL-10 overexpression does not impact the recruitment of immune cells to the gut (**Figure 3D**).

IL-10 Protection against DSS-Induced Colitis Is Not Long Lasting

In our mouse model, IL-10 is also overexpressed in the BM and is found at high levels in the serum, possibly creating an anti-inflammatory environment that could precondition *de novo* generated or circulating monocytes. In this setting, the circulating monocytes could thus be educated to be less responsive once recruited to the colon during DSS-induced colitis. To study this possibility, we combined a period of IL-10 overexpression with a resting period of 7 or 21 days prior to DSS administration (**Figure 4A**). As shown in **Figure 1B**, the levels of IL-10 return
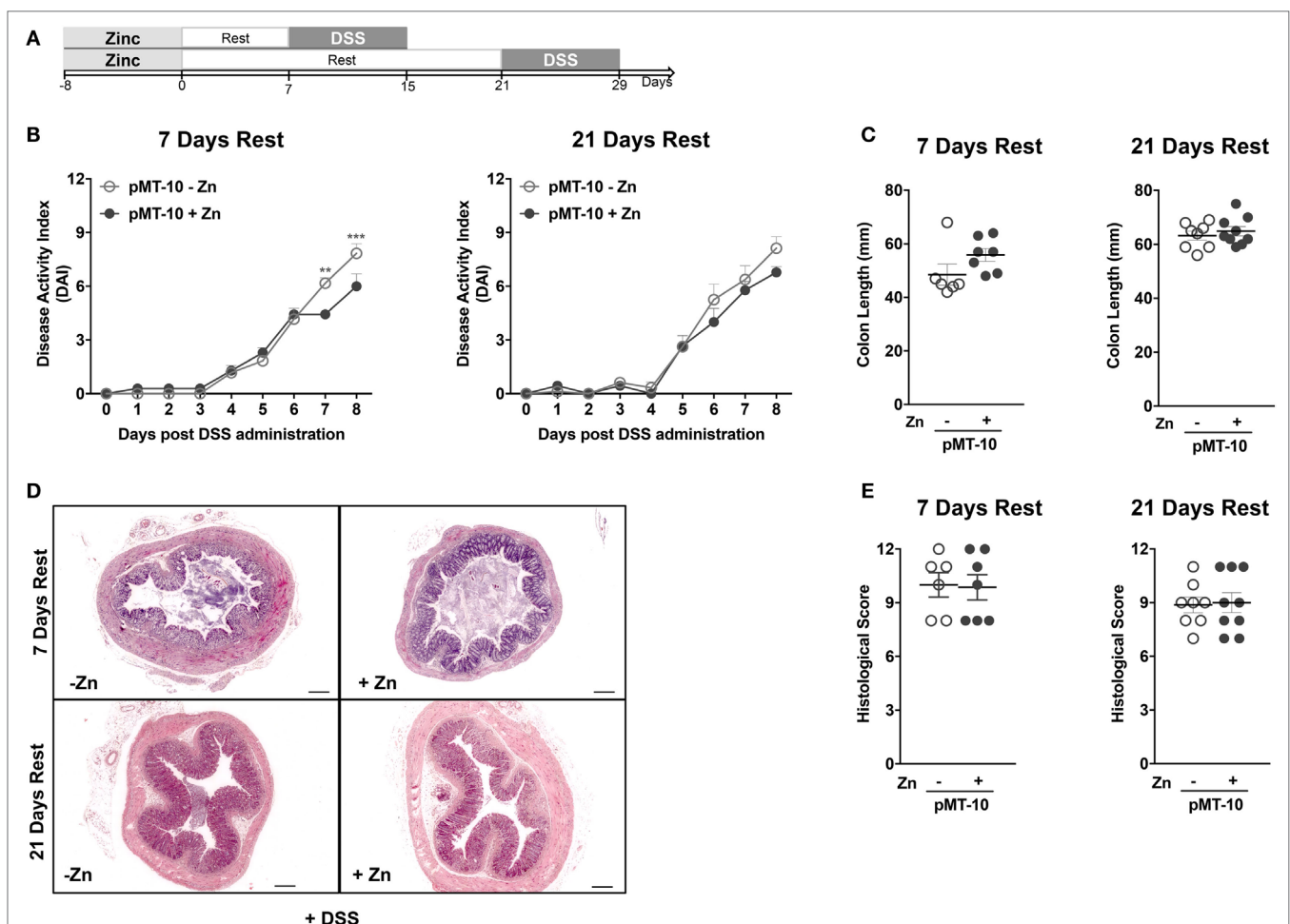
FIGURE 4 | The interleukin-10 protection conferred against DSS-induced colitis is not long lasting. **(A)** pMT-10 mice were fed with control (pMT-10-Zn) or Zn-enriched (pMT-10 + Zn) water for 8 days, followed by a 7- or 21-day resting period where only normal water was available, and by 8 days of 3% DSS. **(B)** Disease progression based on Disease Activity Index (DAI) parameters was registered every day for 8 days. **(C)** Colon length measurement at day 8 of DSS administration. **(D)** Representative H&E-stained sections of large bowel at 40× magnification (scale bar = 200 μm). **(E)** Colitis scores derived from evaluation of colon and cecum from both groups. Each dot represents one independent animal, in two independent experiments; represented is also mean ± SEM. Data were analyzed with **(B)** two-way analysis of variance (Sidak's multiple comparisons test) or **(C,E)** Student's *t*-test.

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to basal ones as soon as 24 h after the zinc administration is suspended. Thus, after a 7-day rest, the circulating levels of IL-10 are normalized. Furthermore, we chose the 21 days time-point, because previous reports showing that resident intestinal macrophages have a life span of approximately 3 weeks (48). After 7-day rest period, the protection in DAI afforded by previous IL-10 exposure was partly lost (**Figure 4B**), being less pronounced than when no resting was performed and only observed at later time-points. After the 21-day resting period, the protection in DAI of pMT-10 previously overexpressing IL-10 was completely ablated (**Figure 4B**). Furthermore, independently of the resting period, no differences were observed between the experimental groups in what concerned colon length (**Figure 4C**) or histology (**Figures 4D,E**). Thus, we conclude that IL-10 overexpression, over a period of 8 days, does not confer long-lasting protection against intestinal inflammation.

DISCUSSION

Despite the fact that IBD is a treatable condition, there are many limitations to the therapeutic approaches currently available (5). Major obstacles in this context are the heterogeneity of the disease, which implies that dosage and schedule may differ across disease conditions, and the requirement of a localized action of the therapeutic agent. In view of the strong immune component associated with disease development, it is not surprising that therapeutic manipulations of the immune response have been widely sought approaches to tackle IBD. Indeed, a commonly used therapy for IBD is the administration of anti-tumor necrosis factor alpha (TNF-α) antibodies (49). However, in line with the above mentioned limitations, up to one-third of IBD patients do not respond to this therapy, and those who respond eventually develop some degree of intolerance to the medication (50). In this context, several animal models of IBD, both spontaneous and experimentally induced (such as DSS), were developed to investigate the role of various factors on the pathogenesis of the disease and to evaluate the different therapeutic options. A molecule that has been widely studied in the context of IBD is IL-10. This cytokine keeps intestinal inflammation in check by exerting a direct effect on monocyte/macrophage populations (34). Thus, it is not surprising that IL-10-based therapies have been tested in IBD. However, both in human (51–54) and mouse models (38), administration of IL-10 did not significantly improve intestinal inflammation, perhaps in part due to the fact that administered IL-10 did not reach the inflamed tissue.

In this study, we report a novel transgenic mouse model of inducible IL-10 overexpression, the pMT-10 mice, in which high IL-10 transcription is observed in the intestine, skin and BM. Upon induction of the transgene, high levels of IL-10 are detected also in the serum. Taking advantage of this novel mouse model, we investigated the dynamics of IL-10 afforded protection during DSS-induced colitis. We found that induction of IL-10 prior to DSS administration impacted the progression of colitis. We show that a short period of IL-10 overexpression before the induction of colitis ameliorated the disease outcome, despite the presence of CD11b+ Ly6C+ cells in the gut, previously associated with the development of detrimental inflammation. However,

in comparison to control animals that do not overexpress IL-10, Ly6C cells isolated from the lamina propria of colitic pMT-10 mice showed a decreased inflammatory profile. Thus, we hypothesize that IL-10 overexpression impairs the response of these cells to the insult, reaffirming both the critical role of these cells on intestinal inflammation (47) and that of IL-10 in regulating their inflammatory responses (34). In line with a previously described protective role for Zn in the context of intestinal inflammation (55, 56), we show some effect of Zn in reducing the severity of colitis, which occurred both later and to a lower extent than that observed for the combined condition Zn + IL-10. In addition, the protective effect of Zn failed to overcome the exacerbated colitis observed in mice that did not respond to endogenous IL-10. The mechanistic bases underlying the protection conferred by Zn alone remain unknown. One possibility is that Zn may contribute to diminish the amount of free radical species generated during acute colitis which contribute to protein, DNA chain and lipid damage (57). In any case, as IBD patients often present a Zn deficiency and respond well to Zn supplementation therapy (58), the exploitation of combined IL-10 and Zn therapies may be worth considering. In line with this, the benefits of combined Zn and anti-TNF therapy were previously described (55).

The pMT-10 mouse model allows for local, as well as systemic, IL-10 overexpression. The fact that we detected increased transcription of the IL-10 transgene in the BM and elevated levels of seric IL-10 led us to hypothesize that preexposure to IL-10 might induce long-lasting transcriptional changes in circulating monocytes, for example through epigenetic imprinting. If this were the case, we might be able to educate these cells to gain long-lasting tolerance to DSS-induced colitis. Our data obtained after a 1- or 3-week rest post-IL-10 exposure show that recent IL-10 exposure is required for maximal protection. Thus, the protective effects of IL-10 were not sustained over time, implying that IL-10 presence at the time of insult is necessary to prevent colitis. Therefore, inducing IL-10 expression in our mouse model at the beginning of disease would be of interest. Unfortunately, we were unable to explore this possibility because the Zn necessary to activate the transgene precipitates in the presence of DSS, when both are provided in the drinking water. In addition, we were unable to induce high levels of IL-10 expression in the serum of mice fed with a Zn-enriched diet and ethical issues prevented us from attempting to induce sustained high levels of IL-10 by frequent gavage or i.p. injections of Zn-containing preparations.

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

ETHICS STATEMENT

In Portugal, all animal experiments were performed in strict accordance with recommendations of the European Union Directive 2010/63/EU and previously approved by Portuguese

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00400/full#supplementary-material.

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Supplementary Material

The Dynamics of Interleukin-10-Afforded Protection during Dextran Sulfate Sodium-induced

Colitis

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A

B
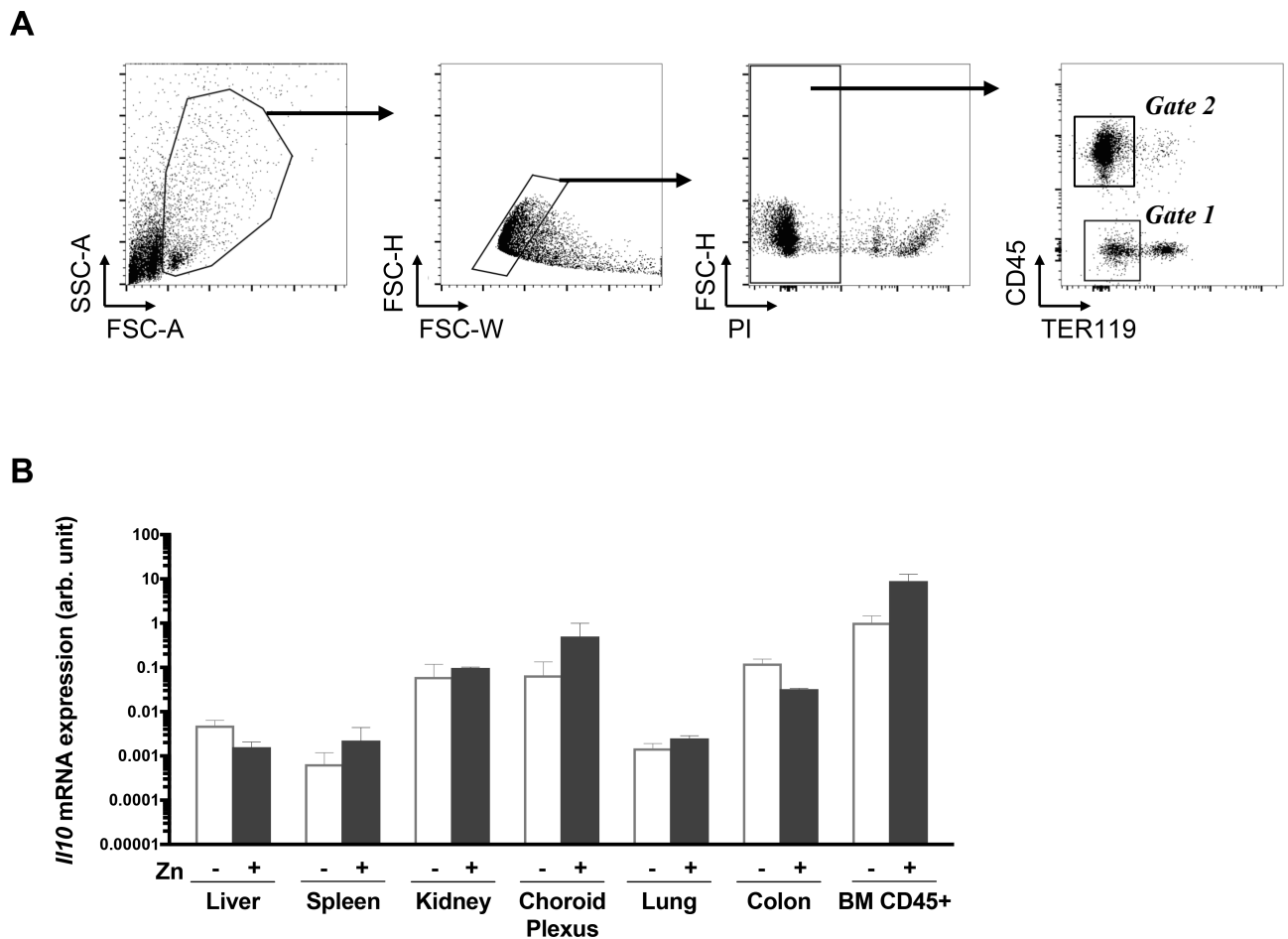
Supplementary Figure 1. (A) Gating strategy for CD45⁻ TER119⁻ cells sort purification. Live non-hematopoietic (CD45-TER119-, gate 1) and hematopoietic (CD45+, gate 2) cells were sort purified based on the lack of expression of CD45 and Ter119. **(B)** Cell suspensions were prepared from the indicated organs as specified in the Methods section, from pMT-10 mice fed with normal (-) or Zn-enriched (+) water. *Il10* mRNA expression was measured by qPCR as indicated in the Methods section.

1
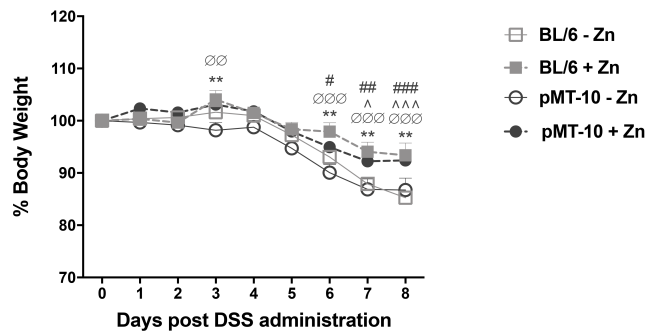
Supplementary Figure 2. Progression of weight loss in DSS-induced BL/6 and pMT-10 mice. BL/6 and pMT-10 mice, fed with control (-Zn) or Zn-enriched water (+Zn), were administered, for 8 days, 3% DSS in the drinking water. Body weight was registered every day for 8 days. Data were analysed with two-way ANOVA (Sidak's multiple comparisons test). # compare BL/6-Zn against BL/6+Zn; ^ compare BL/6-Zn against pMT-10-Zn; $\varnothing$ compare BL/6+Zn against pMT-10-Zn; * compare pMT-10-Zn against pMT-10+Zn. 1 symbol, $p<0.05$; 2 symbols $p<0.01$; 3 symbols, $p<0.001$.

2

Supplementary Table 1. List of Taqman assays used for the BioMark, related to Figure 4.

Probe	Taqman Reference
Hprt	Mm03024075_m1
Gapdh	Mm99999915_g1
Actb	Mm02619580_g1
Il1α	Mm00439620_m1
Il1β	Mm00434228_m1
Il4	Mm00445259_m1
Il6	Mm00446190_m1
Il9	Mm00434305_m1
Il10	Mm01288386_m1
Il12α	Mm00434169_m1
Il12β	Mm01288989_m1
Il13	Mm00434204_m1
Il17	Mm00439618_m1
Il23	Mm0110011_g1
Tnfα	Mm00443258_m1
Ifnγ	Mm01168134_m1
Cxcl1	Mm00436454_m1
Arg1	Mm00475988_m1
Cxcl9	Mm00434946_m1
Cd86	Mm00444543_m1
cMyc	Mm00487804_m1
Flt1	Mm01210866_m1

3

The anti-inflammatory cytokine IL-10 is a new determinant of hematopoietic lineage commitment

Ana Cardoso, A. Gil Castro, Isabel Castro, Ana Cumano, Paulo Vieira and Margarida Saraiva

The anti-inflammatory cytokine IL-10 is a new determinant of hematopoietic lineage commitment

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Graphical Abstract
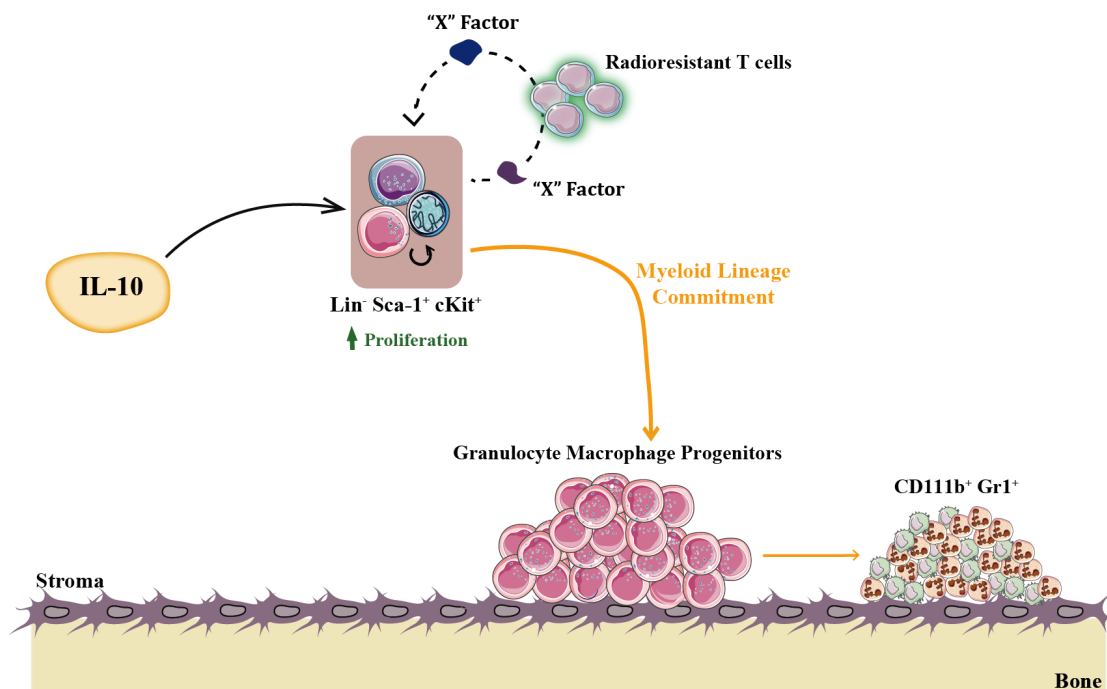
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Abstract

IL-10, a cytokine commonly produced during immune responses, stands out as a major inhibitor of inflammation. Inflammatory cytokines play a recognized role in modulating hematopoiesis to ensure the readjustment of the hematopoietic output to infection. However, and despite the fact that IL-10 production accompanies that of pro-inflammatory cytokines, little is known on whether IL-10 may play a similar role. Using an IL-10 over-expression conditional mouse model (pMT-10), we show that an excess of IL-10 in the organism promotes myelopoiesis, leading to aberrant myeloproliferation and extramedullary hematopoiesis. Furthermore, genetic manipulation of the pMT-10 model combined with reconstitution experiments support a key role for T cells in IL-10 driven myelopoiesis. Finally, through chemical inhibition of intracellular signaling pathways, we report an involvement of PI3K in IL-10-mediated myelopoiesis. Taken together our data places IL-10 as a new factor modulating the hematopoietic output to danger signals.

Introduction

Hematopoiesis is the process that controls the differentiation of hematopoietic cells (HSCs) into lymphoid and myeloid lineages, maintaining the organism homeostasis and conferring protection against pathogens [1-4]. This ability of HSCs to perpetuate through self-renewal and generate new blood cells is regulated by several mechanisms, including signals delivered by the stromal microenvironment [5-7]. These signals regulate proliferation, survival and differentiation of HSCs by activating molecular programs that determine the specification of the precursors along the different lineages [1-4, 8]. Immunologic stress, such as that occurring during neoplasms and infection, changes the magnitude and composition of the hematopoietic output to guarantee proper supply of immune cells to the increased demand, resulting in a process defined as emergency hematopoiesis [9-11]. However, the precise mechanisms that initiate emergency hematopoiesis remain largely unknown.

Interleukin (IL)-10, produced during most of the immune responses, stands out as a major inhibitor of inflammation [12]. During infection, the production of IL-10 is critical to manage the delicate balance between suppressing and activating host responses, hence between the establishment of chronicity or of pathogen clearance, often accompanied by tissue damage detrimental to the host [13, 14]. Understanding the various implications of IL-10 to immune homeostasis is of unquestionable importance, due to potential IL-10 administration for clinical therapy of inflammatory diseases [15]. Indeed, long-term administration of IL-10 may culminate in immunodeficiency, whereas continuous use of anti-IL-10 may lead to hyperimmune reactivities [11, 16-23]. Interestingly, several studies have shown an association between IL- 10 and the pathogenesis of hematopoietic disorders, such as B cell lymphomas [24-27], thus suggesting a possible involvement of IL-10 as a regulator of the hematopoietic process. Altogether, a better understanding of IL-10 potential in regulating hematopoiesis holds the promise to translate into new approaches for treating a variety of human infectious, hematologic, and malignant diseases.

Using a mouse model of inducible IL-10 over-expression (pMT-10) [28, 29] we show that an excess of IL-10 in the organism drives profound hematological alterations, most notably increased myeloid cell production by the bone marrow (BM), development of anemia and extramedullary myelopoiesis with splenomegaly. The hematologic alterations observed required signaling through the IL-10 receptor (IL-10R) complex, since pMT-10 animals deficient for the IL-10Rα chain display a normal phenotype upon induction of IL-10 expression. Further genetic

manipulation of the pMT-10 model combined with reconstitution experiments support a key role for T cells in the mechanism of IL-10 driven myelopoiesis. Finally, through chemical inhibition of intracellular signaling pathways, we report an involvement of the phosphoinositide 3-kinase (PI3K) molecule in IL-10-mediated myelopoiesis. Taken together, our data show that IL-10 over-expression changes the normal hematopoietic output, triggering myelopoiesis. These data add to the complexity of emergency hematopoiesis and to our understanding of hematopoietic deregulation by inflammation and infection.

Material and Methods

Animals

BL/6 mice were purchased from Janvier Labs or Charles River, pMT-10 mice [28], IL-10Rα$^{-/-}$ mice (kindly provided by Werner Müller), pMT-10.IL-10Rα$^{-/-}$ mice (pMT-10^{Tg+} IL-10Rα$^{-/-}$), Rag2$^{-/-}$ mice, Mμ$^{-/-}$ mice, Rag.γc$^{-/-}$ mice (Rag2$^{-/-}$ γc$^{-/- or y/-}$), CD3ε$^{-/-}$ mice, pMT-10.Rag.γc$^{-/-}$ mice (pMT-10^{Tg+} Rag2$^{-/-}$ γc$^{-/- or y/-}$), pMT-10.Rag$^{-/-}$ mice (pMT-10^{Tg+} Rag2$^{-/-}$) and pMT-10.CD3$^{-/-}$ mice (pMT-10^{Tg+} CD3ε$^{-/-}$), were bred either at ICVS, i3S or Pasteur Institute mouse facility under pathogen free conditions.

All animal experiments were done in strict accordance with recommendations of the European Union Directive 2010/63/EU and previously approved by Portuguese National Authority for Animal Health–Direção Geral de Alimentação e Veterinária (DGAV). Mice were euthanized by CO_2 inhalation or cervical dislocation with efforts to minimize suffering.

IL-10 induction

IL-10 over-expression was induced via administration of zinc (Zn) sulfate heptahydrate (Sigma-Aldrich, USA). A solution of 50nM Zn with 2% sucrose was prepared in the drinking water and was deliver to the mice *ad libitum*, during the experimental period [29].

BM Transplantation Assays

Lethally irradiated (850 rad) pMT-10.IL-10Rα$^{-/-}$ 5.2 mice were grafted with 4x10^6 of CD3, TCRβ and TCRγδ-depleted BM cells isolated from BL/6 5.1 mice or Rag.γc$^{-/-}$ 5.1 mice. In cotransfer experiments, BL/6 5.1 BM was injected intravenously (iv) mixed in a 1:1 ratio with BM taken from IL-10Rα$^{-/-}$ CD45.2 mice into pMT-10.IL-10Rα$^{-/-}$ recipients. Recipient mice received 4x10^6 T-depleted BM cells in total. Donor reconstitution was assessed by peripheral blood analysis 4 weeks after transplantation. Sub-lethally irradiated (300 rad) pMT-10.Rag.γc$^{-/-}$ 5.1/5.2 mice were grafted with 4x10^6 BM cells isolated from BL/6 5.2 mice, Rag2$^{-/-}$ 5.1 mice, Mμ$^{-/-}$ 5.2 mice and CD3ε$^{-/-}$ 5.2 mice.

Buparlisib and Ruxolitinib Administration

Ruxolitinib (Ruxo; NVP-CCA0022, Novartis) – a Janus kinase (JAK) 1/JAK2 inhibitor and Buparlisib (Bupa; NVP-BKM120, Novartis) – a PI3K inhibitor, were used to evaluate the signaling pathways activated during IL-10 over-expression. Ruxo and Bupa administration was performed by oral gavage, once daily, during the experimental period. Inhibitors concentration was determined based on previous reports [30-34]. Vehicle solution alone was used on the control groups.

Histology

Samples from the spleen were fixed in 4% paraformaldehyde immediately after mice were sacrificed. The 5μm paraffin-embedded sections were stained with hematoxylin and eosin, and tissue structure was assessed in a blinded fashion.

Preparation of Cell Suspensions

Lymphoid organs – femurs, tibias, thymus and spleens – were recovered into Hank's Balanced Solution (HBSS; Gibco) with 2-4% of FCS. BM cells were extracted by flushing the femurs and tibias with 2mL of HBBS supplemented with 2-4% of FCS. Thymus and spleens were mechanically disaggregated with curved tweezers to obtain single cell suspensions. Cell suspensions were filtered with a 70μm nylon mesh.

Flow Cytometry and Cell Sorting

Cell suspensions were stained with antibodies purchased from eBioscience, Biolegend, BD Bioscience, Sony and Serotec. Briefly, cell suspensions were labeled with lineage (Lin) antibodies (anti-CD3 (145-2C11), anti-CD4 (A161A1), anti-CD8 (53-6.7), anti-CD11c (HL3), anti-CD19 (6D5), anti-B220 (RA3-6B2), anti-Mac1 (M1/70), anti-Gr1 (RB6-8C5), anti-NK1.1 (PK136) and anti-Ter119 (TER119)) for 20min at 4°C and subsequently depleted using Miltenyi Biotec magnetic cell separation system. Remaining, Lin positive (+), cells were identified with Pacific Blue-coupled Streptavidin incubated for 15min at 4°C. The Lin negative (-) fraction obtained was stained with specific antibodies for progenitor markers (anti-IL-7Ra (A7R34), anti-c-Kit (2B8), anti-Sca-1 (D7), anti-FcγR (24G2) and anti-CD34 (RAM34)). Mature populations from BM and spleen were labeled for anti-CD11b (M1/70), anti-CD11c (N418),

anti-Ly6C (HK1.4), anti-Ly6G (1A8), anti-CD64 (X54-5/7.1), anti-Siglec-F (E50-2440), anti-MHC II (M5/114.15.2), anti-F4/80 (CI:A3-1), anti-B220 (RA3-6B2), anti-CD19 (6D5), anti-CD43 (90), anti-IgM (RMM-1), anti-IgD (11-26c.2a), anti-CD3 (145-2C11), anti-CD4 (GK1.5) and anti-CD8 (5H10-1). Antibodies against Ki67, BrdU and Annexin V were also used. Dead cells were excluded with propidium iodide (PI). Stained cells were analyzed using LSR Fortessa, LSR II or Canto II or purified through a FACSAria III (all from BD Biosciences). Cell numbers were estimated using CountBright Absolute Counting beads (Invitrogen) or Cellometer Auto T4 Bright (Nexcelom).

In vitro Cell Cultures

Single cells from common myeloid precursors (CMPs), granulocyte-macrophage precursors (GMPs) or megakaryocyte-erythrocyte precursors (MEPs) subsets were sorted into 60-well Terasaki plates containing 30µl of complete medium (optiMEM supplemented with 20% FCS, Penicillin (50 units/mL), Streptomycin (50 µg/mL) and β-mercaptoethanol (50 µM) all from Gibco,) and supplemented with c-kitL, macrophage (M)- colony stimulating factor (CSF), granulocyte (G)-CSF and granulocyte-macrophage (GM)-CSF, Erythropoietin (Epo) and Thrombopoietin (Tpo). Recombinant cytokines were either purchased from R&D systems or obtained from cell line supernatants. Cultures were supplemented with fresh cytokines at day 3. Frequency scores were assigned based on the frequency of colonies positive wells at day 4 of culture. A day 7 of culture, cells from wells showing colonies were transferred to a slide using a Cytopsin centrifuge at 1000 rpm for 4 min at room temperature. Slides were stained with May-Grünwald for 5min, washed with PBS and stained with Giemsa for 15min. Slides were rinsed with deionized water, and let to dry. Cell morphology was analyzed by microscopy.

RNA extraction, cDNA and classic quantitative real time PCR (qRT-PCR)

mRNA from sorted cells (CMPs, GMPs, MEPs and mature subsets) was extracted using RNeasy Micro kit (Qiagen, 74004) and converted into cDNA by reverse transcription with PrimeScript RT Reagent kit (Takara, Clonetech). qRT-PCR was performed using Taqman primers (*Hprt* - Mm03024075_m1; *CEBPα* - Mm00514283_s1; *EPOR* - Mm00833882_m1; and *Il10* - Mm01288386_m1) and Taqman Universal Master Mix (Applied Biosystems). qRT-PCR reactions were performed on an ABI 7300 thermocycler (Applied Biosystems), gene

expression levels were normalized to Hprt and relative expression was determined using the $2\Delta Ct$ method.

Cytokine Quantification

IL-10 concentration in the serum was quantified using a commercially available ELISA kit (R&D systems). Luminex technology was used to specifically measure IL-10 and G-CSF, GM-CSF, M-CSF, IL-3, IL-1, IFN and TNF, following the manufacturer's instructions (eBioscience).

Statistical Analysis

Statistical analysis was performed with the student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA as indicated in the Figure legends. These tests were performed with Prism Software (GraphPad). Graphs containing errors bars show means $\pm$ SD. Statistical significance is represented as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

IL-10 over-expression associates with increased mature myeloid cells

To investigate the biological impact of IL-10 in hematopoiesis, we resorted to our previously developed and characterized mouse model of IL-10 over-expression, the pMT-10 mice. This mouse model allows for timely controlled IL-10 over-expression in specific anatomic locations, notably in the BM, accompanied by a strong increase of the levels of this cytokine in the serum [29].

For our initial analysis, we have sustained IL-10 expression for 30 days. Serum analysis of IL-10 in induced (Zn-fed) pMT-10 mice showed constant IL-10 over-expression at this time-point, whereas circulating IL-10 was undetectable in pMT-10 mice fed with normal water or in control BL/6 mice (Supplementary Fig. 3.1a). After 30 days of IL-10 over-expression, a striking splenomegaly with increased spleen weight and cellularity, accompanied by a structural disorganization of the tissue were observed (Fig. 3.1a-c). These alterations were not seen in non-induced pMT-10 or BL/6, or in Zn-fed BL/6 (Fig. 3.1a-b), demonstrating that the observed phenotype does not result from pMT-10 genetic background, nor is it driven by Zn administration. Interestingly, the observed increased cellularity was related to a significant increase in myeloid cells, with no major alterations in lymphoid populations (Fig. 3.1d and e and Supplementary Fig. 3.1b). These hematologic alterations required signaling through the IL-10R complex, since induced pMT-10 animals deficient in the IL-10Rα chain displayed a normal phenotype (Fig. 3.1f).

In view of the described alterations, we next hypothesized that the IL-10-driven pronounced alterations in the spleen may result from upstream effects at the hematopoiesis level. To investigate this hypothesis, we performed a detailed analysis of how IL-10 over-expression affected the BM compartment. In line with the spleen data, the BM of induced pMT-10 showed a significant increase in the frequency of the CD11b$^+$Gr1$^+$ population (comprising the CD11b$^+$ Ly6C^{hi} Ly6G$^-$ and CD11b$^+$Ly6C$^+$Ly6G$^+$ subsets) as compared to non-induced pMT-10 mice (Fig. 3.1g). In contrast, a reduction of the frequency of the B cell population was observed in pMT-10 mice fed with Zn-enriched water as compared to pMT-10 fed with control water (Fig. 3.1g).

These hematopoietic changes in the BM and spleen were also reflected in increased percentages of myeloid cells in the blood of induced pMT-10 mice (Supplementary Fig. 3.2a).

Moreover, pMT-10 mice over-expressing IL-10 showed a decreased percentage of B cells and number of red blood cells (RBCs) and platelets in the blood, indicating the occurrence of anemia (Supplementary Fig. 3.2a). Of note, no alterations were observed in the thymus of pMT-10 mice over-expressing IL-10 (Supplementary Fig. 3.2b). These data support the concept that sustained IL-10 exposure impacts on the outcome of the hematopoietic process. Indeed, IL-10 exposure enhanced the myelopoietic output in the BM, and increased the BM and circulating myeloid cell populations with effects on the spleen, cellular composition, size and structure.

IL-10 over-expression alters medullary and extra-medullary hematopoiesis

Considering the substantial changes in mature cell composition imposed by IL-10 over-expression within the BM, we next investigated whether the upstream progenitor populations were also affected. Upon IL-10 over-expression, the cellular composition of the progenitor populations in the BM, suffered remarkable alterations. The percentage of $Lin^- SCA-1^+ cKIT^+$ (LSK) cells in pMT-10 mice over-expressing IL-10 was 10-fold higher than that observed in control mice (Fig. 3.2a). Moreover, a 3-fold increase of the BM GMPs population was also observed in induced pMT-10 mice (Fig. 3.2a). This increase in the GMPs frequency was accompanied by a substantial decrease in CMPs and MEPs in the BM of pMT-10 mice over-expressing IL-10 (Fig. 3.2a). Therefore, the alterations induced by sustained IL-10 exposure in mature myeloid cell populations in the BM seem to result from increased LSK cell frequency and their deviation towards GMPs lineage. These data implicate, for the first time, IL-10 as a modulator of medullary hematopoiesis.

Given the parallel in the phenotype observed for the mature cells in the BM and the spleen, we investigated whether the splenomegaly observed in pMT-10 mice over-expressing IL-10 might be dependent of alterations in the progenitor populations in the spleen. To assess this hypothesis, we analyzed the spleens of control versus pMT-10 mice over-expressing IL-10, for the presence of multipotent and lineage specific hematopoietic progenitors. A significant accumulation of LSK cells was observed in the spleens of induced pMT-10 mice (Fig. 3.2b). In addition, the GMP population, that as expected was very rare in the spleens of control pMT-10 mice, was readily detectable in pMT-10 mice over-expressing IL-10 (Fig. 3.2b). Transcriptional

analysis of the GMPs sort purified from spleens of induced pMT-10 mice showed a profile marked by high levels of *cEBPα* and low expression of *EpoR* (Supplementary Fig. 3.3a). This profile is similar to that of BM-purified GMPs (Supplementary Fig. 3.3a), thus attesting the GMP identity of this splenic population. Furthermore, splenic GMPs of pMT-10 mice over-expressing IL-10 were functionally competent, as they showed the ability to differentiate into mature myeloid cells when cultured in vitro (Supplementary Fig. 3.3b). These results support the occurrence of extramedullary hematopoiesis in the spleens of pMT-10 mice over-expressing IL-10.

The BM is the initial target of IL-10

Our data show an accumulation of LSK cells and GMPs in the BM and spleen after 30 days of IL-10 over-expression. We next sought to investigate the temporal kinetics of the observed IL-10-mediated myeloexpansion. pMT-10 mice over-expressing IL-10 showed a striking increase in the percentage of LSK cells in the BM and spleen as early as day 7 of Zn administration (Fig. 3.2c). Moreover, an increase in the percentage of GMPs in pMT-10 mice over-expressing IL-10 was observed as soon as day 4 of Zn administration (Fig. 3.2c). Next we investigated whether the expansion of LSK cells and GMPs observed in pMT-10 mice over-expressing IL-10 was due to alterations in their proliferative capacity, or associated with altered survival rates. Proliferation and death assays showed that BM LSK cells from pMT-10 mice over-expressing IL-10 for 7 days are proliferating more and dying less than those from control pMT-10 mice (Fig. 3.2d). These observations are compatible with a model where the initial changes driven by IL-10 occur in the BM, where LSK cells proliferate more and differentiate into GMPs even before starting accumulating. Later on, as the spleens of induced pMT-10 mice are able to support extramedullary hematopoiesis, LSK cells may undergo locally differentiation into GMPs, which in turn further increases the numbers of the splenic mature myeloid populations.

Finding that the impact of IL-10 over-expression in hematopoiesis can be detected at LSK and GMPs level as early as day 7, allowed us to perform an additional experiment to further confirm the involvement of IL-10 in this process. Injecting an IL-10-expression plasmid into BL/6 mice mirrored the myeloexpansion observed in induced pMT-10 mice (Supplementary Fig. 3.4). Indeed, as compared to BL/6 mice injected with an empty vector, BL/6 mice injected with the IL-10 plasmid presented an LSK and GMPs expansion in the BM

and spleen, as soon day 7, as observed previously for induced pMT-10 mice (Supplementary Fig. 3.4).

The IL-10-driven myeloexpansion requires IL-10R signaling in the hematopoietic compartment, and occurred via direct and indirect effects

The myeloexpansion observed in pMT-10 mice requires IL-10R signaling (Fig. 3.1f). Most hematopoietic cells, including stem and progenitor subsets, express the IL-10R [35], thus being potential targets of IL-10. However, IL-10 cellular target and whether the observed effects result from a direct or indirect action of IL-10 remain unknown. To start dissecting the mechanisms underlying the IL-10-driven myeloexpansion in pMT-10 mice we used a BM transfer model. In this model, irradiated recipient pMT-10.IL-10R$\alpha^{+/+}$ mice or pMT-10.IL-10R$\alpha^{-/-}$ mice were reconstituted with T cell-depleted BM from BL/6 CD45.1 mice (Fig. 3.3a). After reconstitution, the chimeric mice were fed with Zn-enriched water for 15 days, at which time the presence of precursor cell populations was quantified in the BM or spleen. Data from the chimeric mice were compared to control pMT-10 mice. Independently of the presence or absence of IL-10R in the recipient mice, an expansion of both LSK and GMP populations and a contraction of CMP precursors were observed in the BM (Fig. 3.3b) and the spleen (Fig. 3.3c). These observations indicate that aberrant myeloexpansion triggered by IL-10 required IL-10Rα signaling in hematopoietic cells, and not in stromal cells, as the recipient mice were IL-10Rα deficient.

To further understand whether the expansion of the progenitor populations was solely and directly mediated by IL-10, we performed a second set of immune reconstitution experiments. Irradiated pMT-10.IL-10R$\alpha^{-/-}$ mice were reconstituted with a mixture of T cell-depleted BM obtained from BL/6 5.1 and IL-10R$\alpha^{-/-}$ 5.2 mice (Fig. 3.4a). As before, after reconstitution, the mice were fed with Zn-enriched water for 15 days and the LSK and GMPs cell populations present in their BM and spleens analyzed as proxies for the IL-10-driven phenotype (Fig. 3.4b and c). Due to the differential expression of CD45.1 and CD45.2 in donor BL/6 wild type or IL-10R$\alpha^{-/-}$ mice, respectively, we were able to determine the origin of the expanding populations. Both in the BM (Fig. 3.4b, d-e) and the spleen (Fig. 3.4c, f-g), the expansion of LSK cells in the reconstituted mice was decreased, while that of GMPs was unaffected, as compared to control

induced pMT-10 mice. Whereas in the BM no significant changes were seen in the proportion of IL-10R-competent versus -deficient LSK cells (Fig. 3.4d), in the spleen, IL-10R-competent LSK cells proliferated more than IL-10R-deficient ones (Fig. 3.4f). These data indicate that both a direct and an indirect effect of IL-10 on hematopoietic cells might be in place and that LSK cells are likely more dependent on the IL-10R signaling than GMPs.

T cells are required for the IL-10-driven myeloexpansion

We next sought to investigate IL-10 primary cell population target, among hematopoietic cells. For this, we generated pMT-10.Rag.γc$^{-/-}$ mice and induced IL-10 over-expression. Of note, Rag$^{-/-}$ mice have impaired T and B-cell differentiation due to the absence of antigen receptors while deficiency in the γc receptor blocks differentiation of natural killer (NK) cells and innate lymphoid cells (ILCs) [36, 37]. Induced pMT-10.Rag.γc$^{-/-}$ mice showed no hematopoietic alterations in any of the tested progenitor cell subsets, presenting a BM and spleen comparable to control pMT-10 mice (pMT-10-Zn) (Fig. 3.5a-d). These results suggest that a lymphoid cell population plays an essential role in the IL-10-driven myeloexpansion.

To investigate which hematopoietic populations are targeted by IL-10, we resorted to another set of reconstitution experiments. Irradiated pMT-10.Rag.γc$^{-/-}$ mice were reconstituted with either T-depleted BM M$\mu$$^{-/-}$ mice or total BM of Rag$^{-/-}$ mice (Fig. 3.6a and b). As controls we used pMT-10 fed with normal or Zn-enriched water, and irradiated pMT-10.IL-10R$\alpha$$^{-/-}$ mice reconstituted with Rag.γc$^{-/-}$ BM cells (Fig. 3.6c). pMT-10.Rag.γc$^{-/-}$ mice reconstituted with M$\mu$$^{-/-}$ showed an expansion of both LSK cells and GMPs population in BM and spleen after 15 days of IL-10 over-expression (Fig. 3.6a and Supplementary Fig.5a and b). Mice reconstituted with Rag$^{-/-}$ BM cells showed no LSK or GMPs expansion either in the BM or spleen when compared to induce pMT-10 mice (Fig. 3.6b and Supplementary Fig.5a and b). These results exclude B cells as IL-10 target cells, whilst suggesting a contribution of T cells to the IL-10-induced myeloproliferation. Surprisingly, pMT-10.IL-10R$\alpha$$^{-/-}$ mice reconstituted with Rag.γc$^{-/-}$ BM cells and over-expressing IL-10 mirrored the phenotype developed by control pMT-10 over-expressing IL-10 (Fig. 3.6c). Indeed, induced pMT-10.IL-10R$\alpha$$^{-/-}$ mice reconstituted with Rag.γc$^{-/-}$ cells presented a marked increase of the GMPs population both in the BM and spleen when compared to control pMT-10 mice (Fig. 3.6c). This was unexpected, as we had previously shown that pMT-10.Rag.γc$^{-/-}$ over-expressing IL-10 failed to develop the myeloexpansion

phenotype (Fig. 3.5a). Together, these observations suggest the involvement of a radioresistant T cell subset in IL-10 driven myeloexpansion, as T cells are the only remaining lymphoid population in the induced pMT-10.IL-10Rα$^{-/-}$ mice reconstituted with Rag.γc$^{-/-}$ cells (data not shown).

To further confirm the role of T cells in IL-10 driven phenotype, we crossed pMT-10 with CD3ε$^{-/-}$ mice and induced IL-10 over-expression in both pMT-10.CD3$^{+/-}$ and pMT-10.CD3$^{-/-}$ mice. pMT-10.CD3$^{-/-}$ mice over-expressing IL-10 showed no hematopoietic alterations in any of the tested progenitor cell subsets in the BM and spleen, while its littermate control, pMT-10.CD3$^{+/-}$ mice, developed the characteristic LSK and GMP expansion of pMT-10 mice (Fig. 3.7a-d). Together, these set of experiments suggest that T cells are required for the IL-10-driven myeloexpansion, although not being IL-10 first cell target.

Signaling cascades mediating IL-10 induced myelopoiesis

Among the well-described signaling cascades triggered upon activation of the IL-10R are the JAK/ Signal transducers and activators of transcription (STAT) and PI3K/AKT/ Glycogen synthase kinase 3 (GSK3) pathways [38, 39]. Importantly, both JAK/STAT and PI3K signaling cascades have been implicated in aberrant myelopoiesis, such as during myeloproliferative disorders [40-43].

To investigate if JAK/STAT and PI3K cascades were involved in the IL-10-driven myeloexpansion, we resorted to specific JAK1/2 (Ruxo) and PI3K (Bupa) inhibitors. We questioned whether these inhibitors reverted the myeloexpansion developed upon IL-10 over-expression. For this, we induced IL-10 over-expression for 30 days, followed by 7 days of Ruxo and/ or Bupa treatment in parallel with Zn administration in the drinking water. Treatment with Ruxo, Bupa or Ruxo+Bupa impacted the outcome of sustained IL-10 over-expression in pMT-10 mice. Animals treated with Bupa or Ruxo+Bupa showed a reduction in spleen weight and size, with a recovery of the spleen histological structure (Fig. 3.8a and b). Treatment with Ruxo was not as efficient, with a less marked reduction of the spleen weight and size, and no signs of recovery of the spleen structural organization (Fig. 3.8a and b).

BM analysis revealed a reduction of the LSK compartment in all treated animals. Percentage of LSK cells in Ruxo, Bupa and Ruxo+Bupa treated groups was decreased when compared to the group pMT-10+Zn, but still higher than the pMT-10-Zn (Fig. 3.8c). Both these

control groups were treated with vehicle solution. Additionally, GMPs showed no alterations when compared to non-treated pMT-10 mice over-expressing IL-10 (Fig. 3.8c). These observations were reflected in the spleens of treated mice, as no alterations in the LSK population were observed when compared to pMT-10+Zn (Fig. 3.8d). Splenic GMPs remained altered in all treated groups (Fig. 3.8d). Further analysis of the different mature subsets revealed a slight reduction of BM monocytes (CD11b$^+$Ly6C^{hi}Ly6G) in Bupa and Ruxo+Bupa treated groups, in comparison to the pMT-10+Zn (Fig. 3.8e). However, no differences were observed in the neutrophil subset (CD11b$^+$Ly6C^{int}Ly6G$^+$) in the BM (Fig. 3.8e). Spleen analysis of Bupa and Ruxo+Bupa treated groups, showed a decrease of the monocyte subsets that paralleled the one observed the BM (Fig3.8f). Interestingly, the effect of Bupa and Ruxo+Bupa in splenic neutrophils was obvious, as the treated mice showed a marked decrease of the CD11b$^+$Ly6C^{hi}Ly6G$^-$ population when compared to pMT-10+Zn (Fig.3.8f). Of note, BL/6 fed or not with Zn, treated with Ruxo+Bupa Zn showed a marked reduction in the percentage of CD11b$^+$Ly6C^{hi}Ly6G$^-$ cells in the BM but no alterations in the neutrophil subset (Supplementary Fig. 3.7a). Moreover, no alterations were observed in the spleen of treated BL/6 mice (Supplementary Fig. 3.7b).

In all, these data demonstrates that JAK and PI3K inhibition allows for partial recovery of the spleen structure, with a more pronounced effect being observed with PI3K inhibitors. However, these inhibitors do not allow for recovery at the progenitor level, thus likely compromising a long-term positive effect.

Discussion

Hematopoiesis ensures the daily replenishment of most mature blood cells. In addition to this homeostatic cellular amplification and differentiation, the hematopoietic system is capable of adapting its cellular output in response to stress, for example during infection or chemotherapy recovery. This process, generally termed emergency hematopoiesis, is regulated through a complex network of molecules and results in an increase output of myeloid cells [44]. Among the signals triggering emergency hematopoiesis are the CSF M-CSF, G-CSF and GM-CSF, and several cytokines, as type I and type II interferon (IFNs), IL-1β and IL-6 [44]. We here propose a previously unappreciated contribution of the anti-inflammatory cytokine IL-10 in reprograming hematopoiesis, leading to a greatly increased myeloid cell output.

Previous studies suggested a role for IL-10 in hematopoiesis. HSCs stimulation with IL-10 enhanced their self-renewal potential [45] and an increase of myeloid progenitor cells was observed when CD34+ cells were cultured with the classical cocktail for cell survival supplemented with recombinant IL-10 [46]. Furthermore, several reports have shown an association between IL-10 and the pathogenesis of hematopoietic disorders, such as B cell lymphomas [24-27]. To the best of our knowledge, our study is the first to report the impact of IL-10 in hematopoietic cell commitment in an *in vivo* model and to provide evidence on the underlying mechanisms, despite some still outstanding questions.

Considering that IL-10 is produced by many immune cells in response to various stimuli [12], the presence of this cytokine in the BM niche during infection or other stress signals is not unexpected. Our findings are compatible with a mechanism wherein IL-10 acts at the BM level, initiating an expansion of LSK cells. This response of the hematopoietic niche to IL-10 is independent of IL-10R signaling in the stroma, thus indicating that a hematopoietic cell is the initial target of IL-10. During emergency hematopoiesis, myeloid-restricted progenitor cells normally identified as Lin$^-$Sca-1$^-$c-Kit$^+$, become positive for Sca-1 and can no longer be distinguished from the real multipotent population of LSK, often resulting in an overestimation of the latter population. Thus, to exclude this possibility and confirm the authenticity of the expanded BM LSK population in pMT-10 mice over-expressing IL-10, a more detailed FACS analysis of the progenitor cells, including the surface markers CD34, CD48, CD150 and Flt3, is required. These markers will allow us to identify the different progenitor subsets enclosed in

the normal LSK compartment and reveal which particular LSK population is expanded in the presence of IL-10. This will be of utmost importance for the performance of more detailed molecular studies focused in the cellular population mostly affected by IL-10, by single cell RNA *Seq*, elucidating how the presence of IL-10 alters the transcriptional identity of progenitor cells. On-going studies are now being performed to evaluate if the presence of IL-10 impacted on the LSK capacity to give rise to all cells from the different lineages through *in vitro* assays.

Notably, LSK cells express the surface receptor for IL-10 [35], attesting their ability to respond to IL-10. Nevertheless, IL-10 on its own may not be enough to promote LSK expansion, as in the absence of T cells this phenotype is lost. We thus hypothesize that in response to IL-10 another molecular mediator is required to instruct the downstream T cell response. Importantly, our experiments with reconstituted pMT-10.IL-10Rα$^{-/-}$ mice show that T cells are not responding directly to IL-10, hence the need of an intermediate signal. In response to this IL-10-dependent signal, T cells further alter the hematopoietic niche, favoring myelopoiesis. Further studies, as depletion of the CD4 and CD8 populations in induced pMT-10 mice, and reconstitution of pMT-10.CD3$^{-/-}$ mice with BM depleted in TCRαβ or TCRγδ cells will help elucidate the missing links in this mechanism. Once a target T cell population is found, we will purify it from induced and non-induced pMT-10 mice and perform RNA-Seq. This will indicate what alterations are seen in T cells upon exposure to IL-10, which in turn will offer candidates bridging IL-10 and T cell responses, as well as linking T cell responses and myelopoiesis.

Interestingly, the reported effects of IL-10 are reversible, as once Zn administration is suspended and the IL-10 expression returns to basal levels, we observe a retraction of myelopoiesis to homeostatic levels (Supplementary Fig. 3.8a-c). This indicates that transient administration of IL-10 does not lead to long lasting cellular transformation, but instead transiently deregulates the system.

Despite these still unanswered questions in what regards the mechanistic details of IL-10-driven myelopoiesis, our study bares unquestionable important implications. The immune response is shaped by the ability of the hematopoietic system to expand and contract populations of myeloid and lymphoid lineages upon different stimuli. Over the past years, a special attention has been given to the factors that modulate the cell output during emergency

hematopoiesis. Our findings now place IL-10 in this circuit, revealing how complex the hematopoietic process really is. We further offer T cells as important mediators of myelopoiesis. It is tempting to speculate that T cells may also be involved in emergency hematopoiesis during for example infection, a fascinating hypothesis that awaits clarification.

In summary, our study enhances our understanding of the complex regulatory links operating in hematopoiesis, unlocking IL-10 as a regulator of myeloid cell differentiation, in addition to being a regulator of myeloid cell responses. These data thus illustrate the intertwining and complexity of the immune system functioning.

Author Contributions

AC: conceptual and experimental design, execution of the project, analysis, interpretation and discussion of the data and manuscript writing; AGC: interpretation and discussion of the data and critical manuscript revision; IC: discussion of the data and critical manuscript revision; AC: conceptual and experimental design, interpretation and discussion of the data and critical manuscript revision; PV: conceptual and experimental design, interpretation and discussion of the data and critical manuscript writing; MS: conceptual and experimental design, interpretation and discussion of the data and critical manuscript writing.

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Figure Legends

Fig 3.1. pMT-10 mice over-expressing IL-10 present splenomegaly, spleen histological disorganization and increased cellularity. Normal or Zn-enriched water was fed to BL/6, pMT-10 and pMT-10.IL-10Rα^{-/-} mice for 30 days. At day 30, **(a)** spleen size and **(b)** total number of leukocytes of pMT-10 mice fed with normal (pMT-10-Zn) or Zn-enriched (pMT-10+Zn) water was recorded. **(c)** Representative images of each group spleen size and histology. **(d)** Numbers of splenic DCs (CD11b^{-} CD11c^{+}), eosinophils (CD11b^{+} Siglec-F^{+}), monocytes (CD11b^{+} Ly6C^{hi} Ly6G^{-}) and neutrophils (CD11b^{+} Ly6C^{+} Ly6G^{+}) were assessed in both groups. **(e)** Numbers of B cells (B220^{+} CD19^{+}), T cells (CD3^{+}). **(f)** Spleen weight, total number of leukocytes and total number of monocytes/ neutrophils (CD11b^{+} Gr1^{+}) in pMT-10.IL-10Rα^{-/-} mice fed with Zn-enriched water and respective controls. **(g)** Representative plots and statistics of the mature populations in the BM of pMT-10-Zn or pMT-10+Zn. Each bar represents the Mean±SD for 3-6 independent mice, in 2 independent experiments. Data were analyzed with student's t-test **p<0.01; ***p<0.001.

Fig 3.2. Myeloexpansion in the BM of pMT-10 mice is readily observed after 4 days of IL-10 induction. (a) Normal or Zn-enriched water was fed to pMT-10 mice for 30 days. At day 30, after lineage depletion, cell suspensions were stained for IL-7Rα, cKIT, SCA-1, FcγR, CD34 and Flt3. Representative plots of BM LSK (Lin^{-} cKit^{+} Sca-1^{+}), CMPs (Lin^{-} IL-7Rα^{-} c-Kit^{lo} Sca-1^{-} FcγR^{lo} CD34^{+}), GMPs (Lin^{-} IL-7Rα^{-} c-Kit^{+} Sca-1^{-} FcγR^{+} CD34^{hi}) and MEPs (Lin^{-} IL-7Rα^{-} c-Kit^{+} Sca-1^{-} FcγR^{-} CD34^{lo}) and statistics in both groups. **(b)** Representative plots and statistics of LSKs and myeloid precursor populations in the spleen of pMT-10-Zn and pMT-10+Zn after 30 days of Zn. **(c)** Kinetics of IL-10 driven myeloexpansion. The cellular composition of the BM and the spleen was determined on days 0, 4, 7, 14 and 30 of IL-10 induction. Each point or bar represents the Mean±SD for 3-6 independent mice, in 2 independent experiments. **(d)** At day 7 of induction, proliferation and survival of the different progenitor populations, in pMT-10-Zn and pMT-10+Zn, was evaluated using Ki67 and Annexin V. Data were analyzed with **(a, b and d)** student's t test or **(c)** two-way ANOVA (Sidak's multiple comparisons test) *p<0.05; **p<0.01; ***p<0.001.

Fig 3.3. The IL-10-driven myeloexpansion requires IL-10R signaling in the hematopoietic compartment. (a) Transplantation assays were performed by

injecting 4×10^6 T cell-depleted BM donor cells from BL/6 5.1 mice into lethally irradiated pMT-10.IL-10Rα$^{+/+}$ or pMT-10.IL-10Rα$^{-/-}$ mice. Five weeks after reconstitution Zn was administered to the animals for two weeks. As controls, non-induced pMT-10 mice were used. Progenitor cell populations in the **(b)** BM and (c) spleen were assessed at 15 using the same cell surface markers as before. Representative plots and statistics of LSKs and myeloid precursor populations in the **(b)** BM and **(c)** spleen of each group. Each bar represents the Mean±SD for 3-6 independent mice, from one representative experiment. **(b-c)** Data were analyzed with one-way ANOVA *p<0.05; **p<0.01; ***p<0.001.

Fig 3.4. The IL-10-driven myeloexpansion occurs via direct and indirect effects. (a) Transplantation assays were performed by injecting 4×10^6 T cell-depleted BM donor cells from BL/6 5.1 or IL-10Rα$^{-/-}$ 5.2 mice into lethally pMT-10. IL-10Rα$^{-/-}$ mice. Five weeks after reconstitution, Zn was administered to the animals for two weeks. Progenitor cell populations in the **(b)** BM and **(c)** spleen were assessed at day 15 using the same cell surface markers as before. Representative plots **(b-c)** and statistics **(d-g)** of LSKs and GMP populations in the **(b, d, e**) BM and **(c, f, g)** spleen of each group. Each bar represents the Mean±SD for 3-6 independent mice, from one representative experiment. **(d-g)** Data were analyzed with one-way ANOVA or student's t test *p<0.05; **p<0.01; ***p<0.001.

Fig 3.5. Lymphoid cells mediate IL-10-driven myeloexpansion. Normal water or Zn-enriched water was administered to pMT-10 and pMT-10.Rag.γc$^{-/-}$ mice for 15 days. **(a-b)** At day 15, progenitor subsets were assessed as before, both in the BM and spleen. **(c-d)** Representative plots and statistics of LSKs and myeloid precursor populations of each group. Each point or bar represents the Mean±SD for 3-6 independent mice, from one representative experiment. **(c-d)** Data were analyzed with one-way ANOVA **p<0.01; ***p<0.001.

Fig 3.6. Radioresistant lymphoid cells mediate IL-10 effects in pMT-10 mice. Transplantation assays were performed by injecting either: **(a)** 4×10^6 T cell-depleted Mμ$^{-/-}$ BM cells, **(b)** total BM of Rag$^{-/-}$ mice into pMT-10. Rag.γc$^{-/-}$ mice or **(c)** irradiated pMT-10.IL-10Rα$^{-/-}$ mice reconstituted with Rag.γc$^{-/-}$ BM cells. As controls for all reconstituted group we used pMT-10 fed with normal or Zn-enriched water, and 5
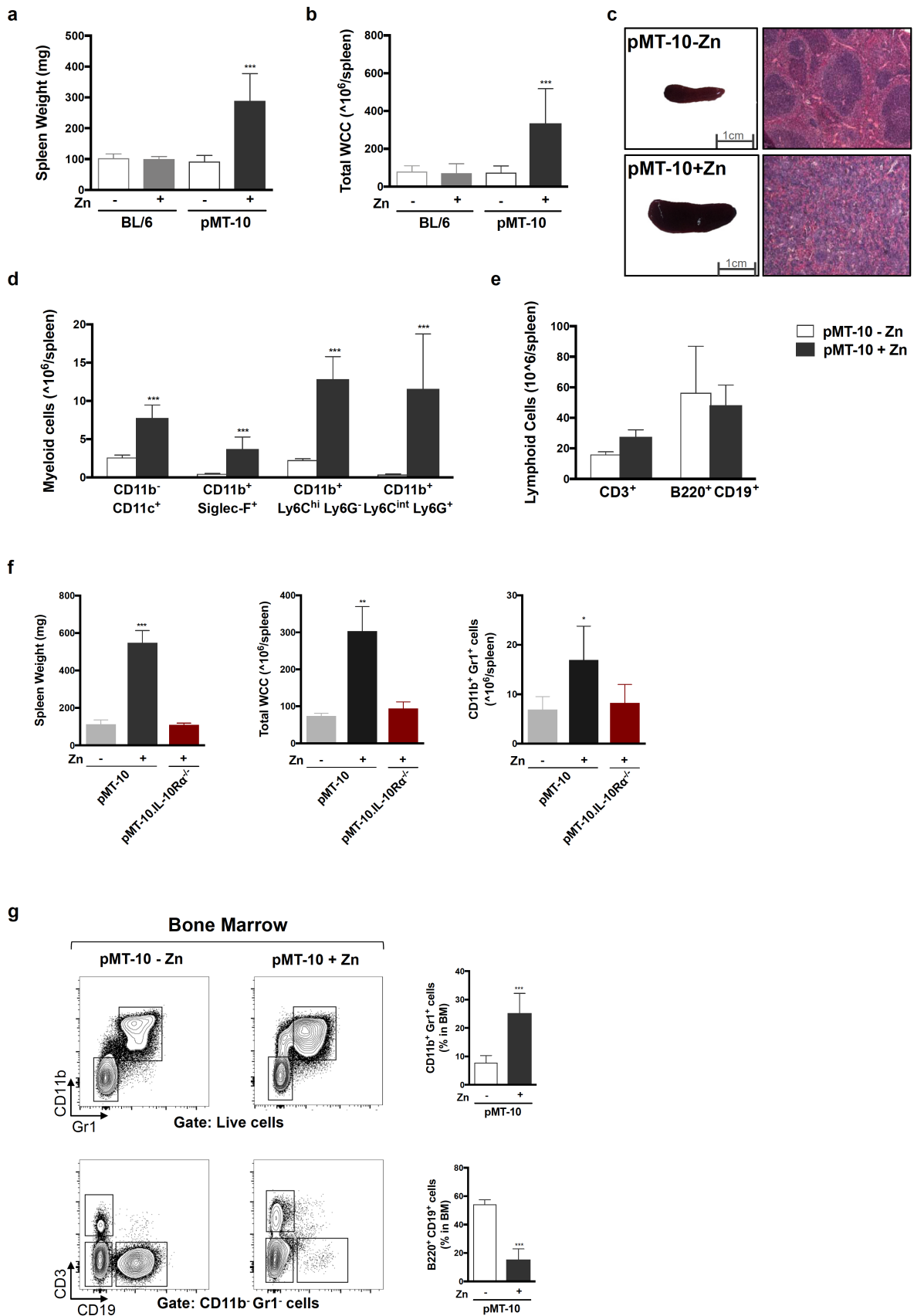
weeks after irradiation and reconstitution, Zn was administered to the animals for two weeks. Progenitor populations in the BM and spleen were assessed at day 15 using the same cell surface markers as before. Each point or bar represents the Mean±SD for 3-6 independent mice, from one representative experiment. **(a-c)** Data were analyzed with one-way ANOVA *p<0.05; **p<0.01; ***p<0.001.

Fig 3.7. T cells mediate IL-10-driven myeloexpansion. Zn-enriched water was administered to pMT-10.CD3$^{+/-}$ and pMT-10.CD3$^{-/-}$ mice for 15 days. **(a-b)** At day 15, progenitor subsets were assessed as before, both in the BM and spleen. **(c-d)** Representative plots and statistics of LSKs and myeloid precursor populations of each group. Each point or bar represents the Mean±SD for 3-6 independent mice, from one representative experiment. **(c-d)** Data were analyzed with one-way ANOVA) **p<0.01; ***p<0.001.

Fig 3.8. JAK2 and PI3K inhibitors attenuate effects of IL-10 over-expression. IL-10 over-expression in pMT-10 mice was induced for 30 days. At day 30, inhibitors or vehicle were administrated by oral gavage in parallel with Zn in the drinking water. (a) At day 37, BM and spleens were harvested and their weight determined. (b) Histological analysis was done with HE staining. (c-f) Leucocyte populations were assessed using the same cell surface markers as before, for both progenitors and mature subsets in the BM and spleen. Data from two experiments, with Mean±SD for 6 independent mice. Data were analyzed with one-way ANOVA *p<0.05; **p<0.01; ***p<0.001.
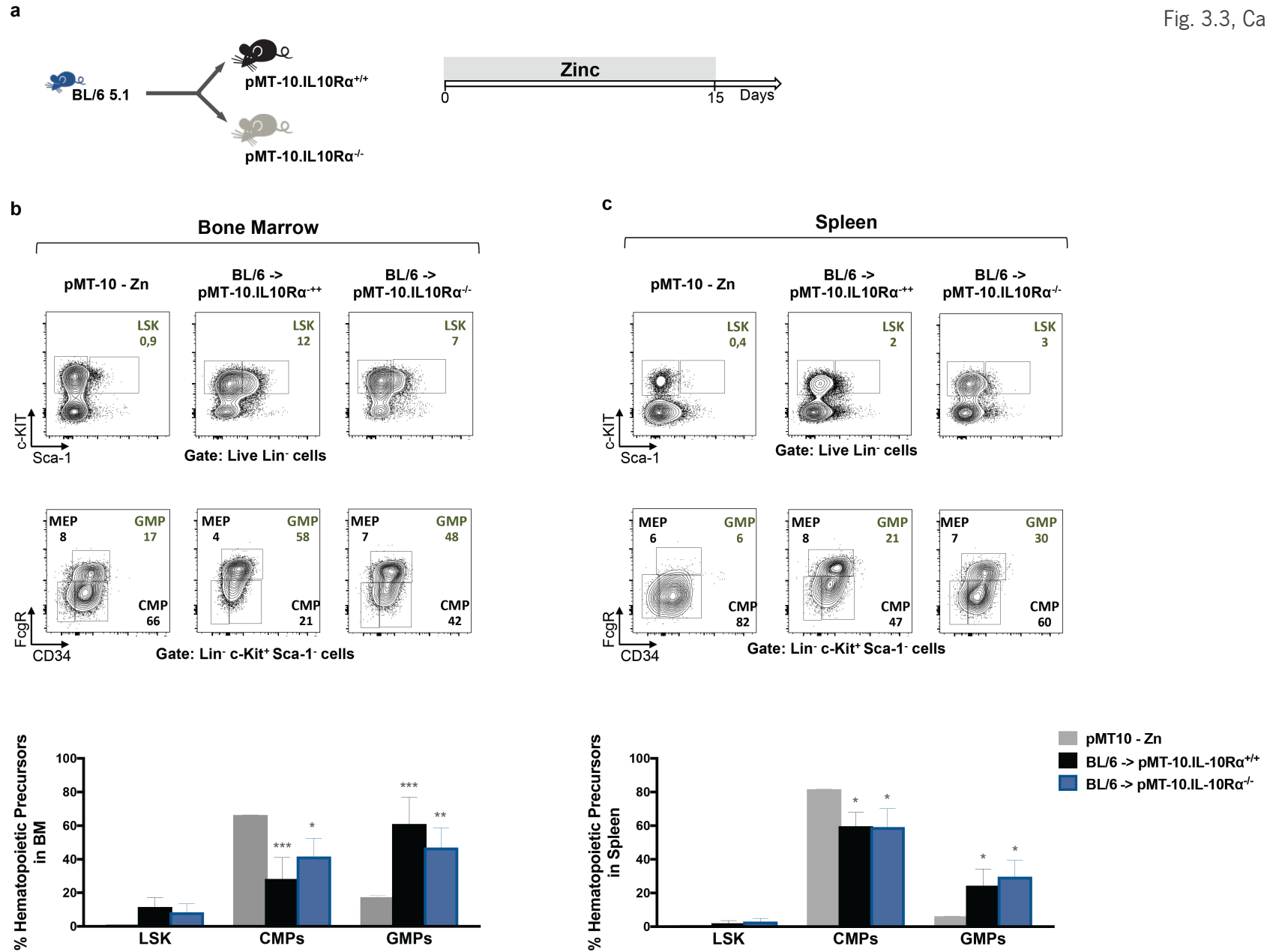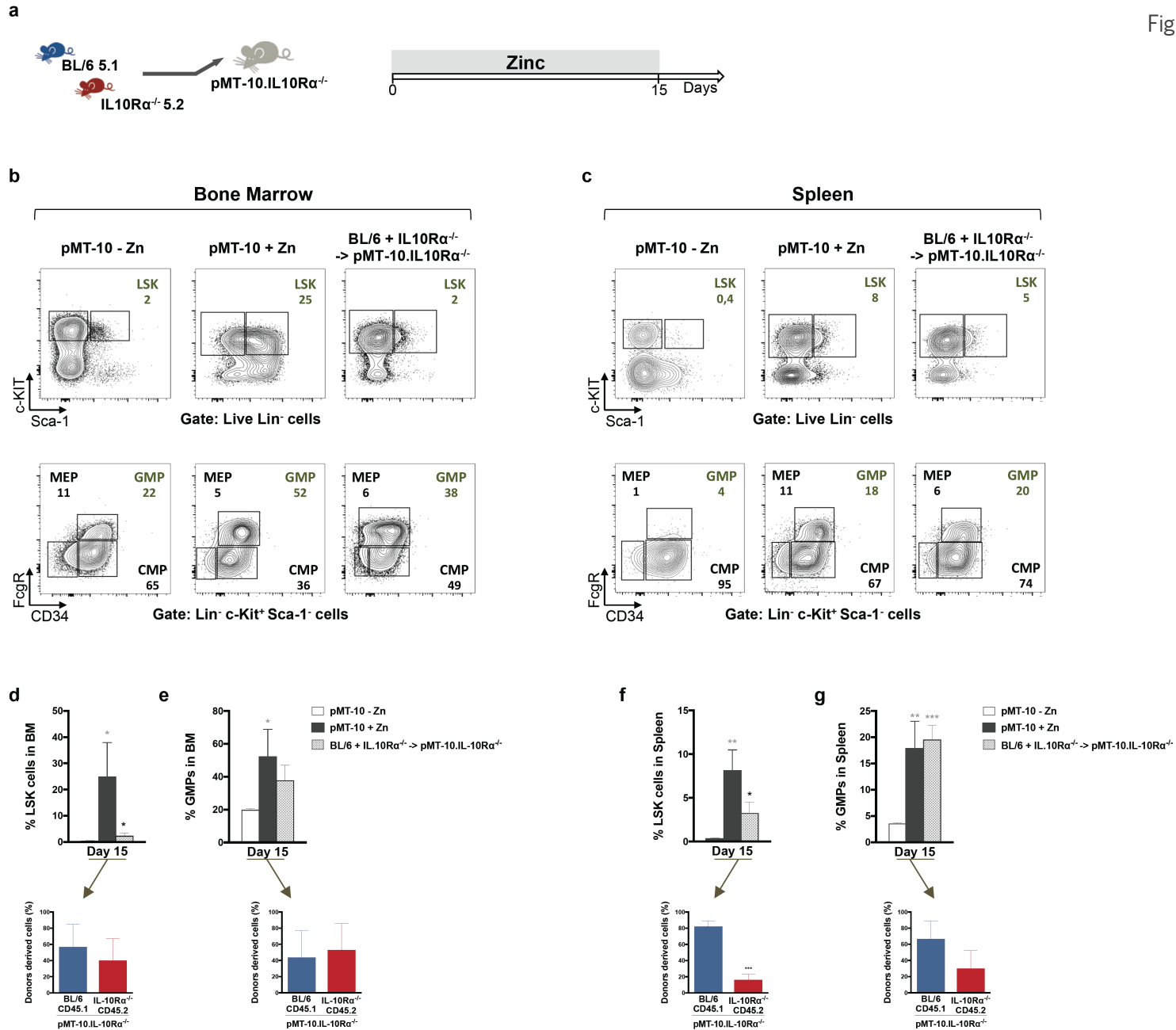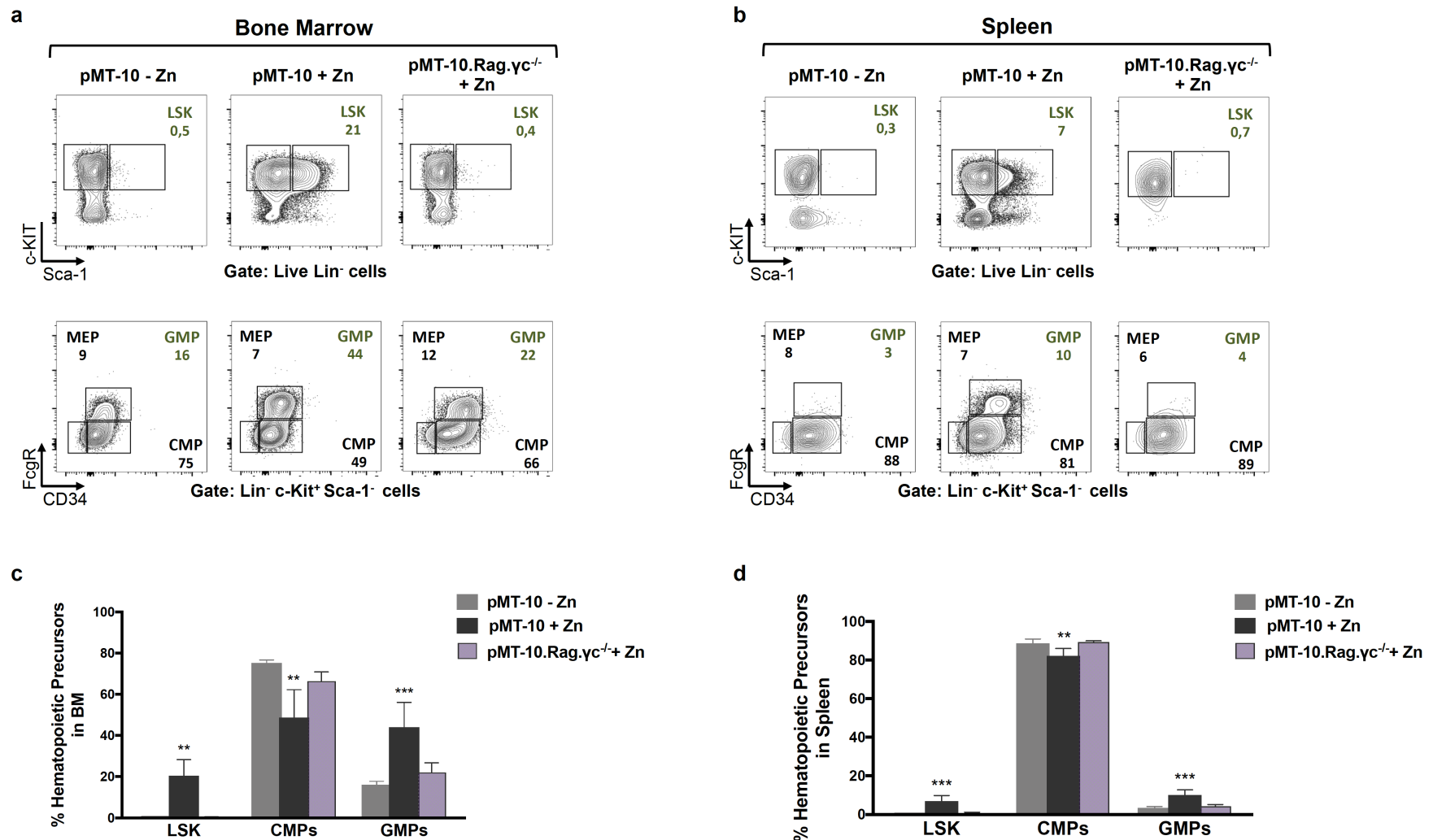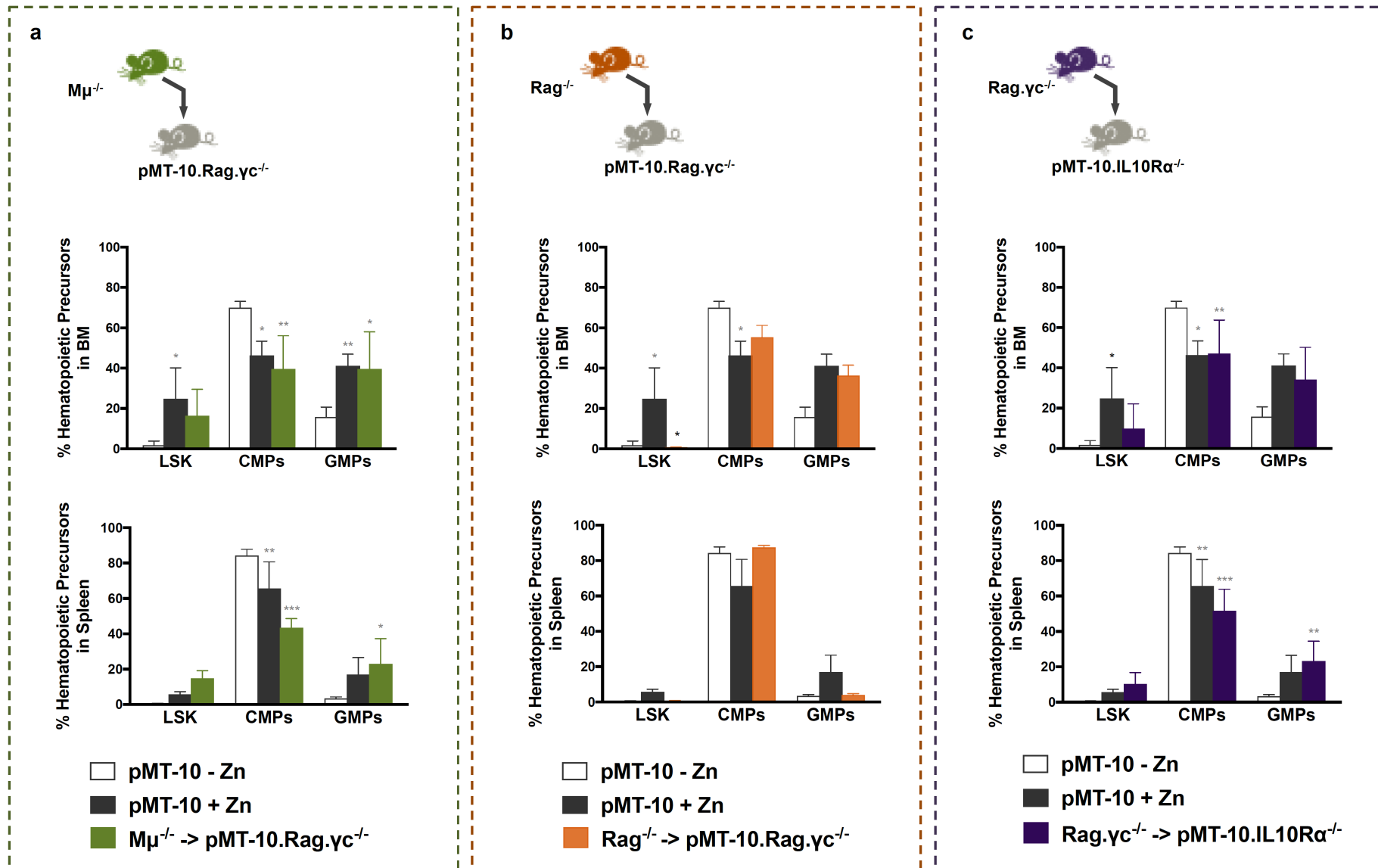
Figures

a

b Bone Marrow

c Spleen

a
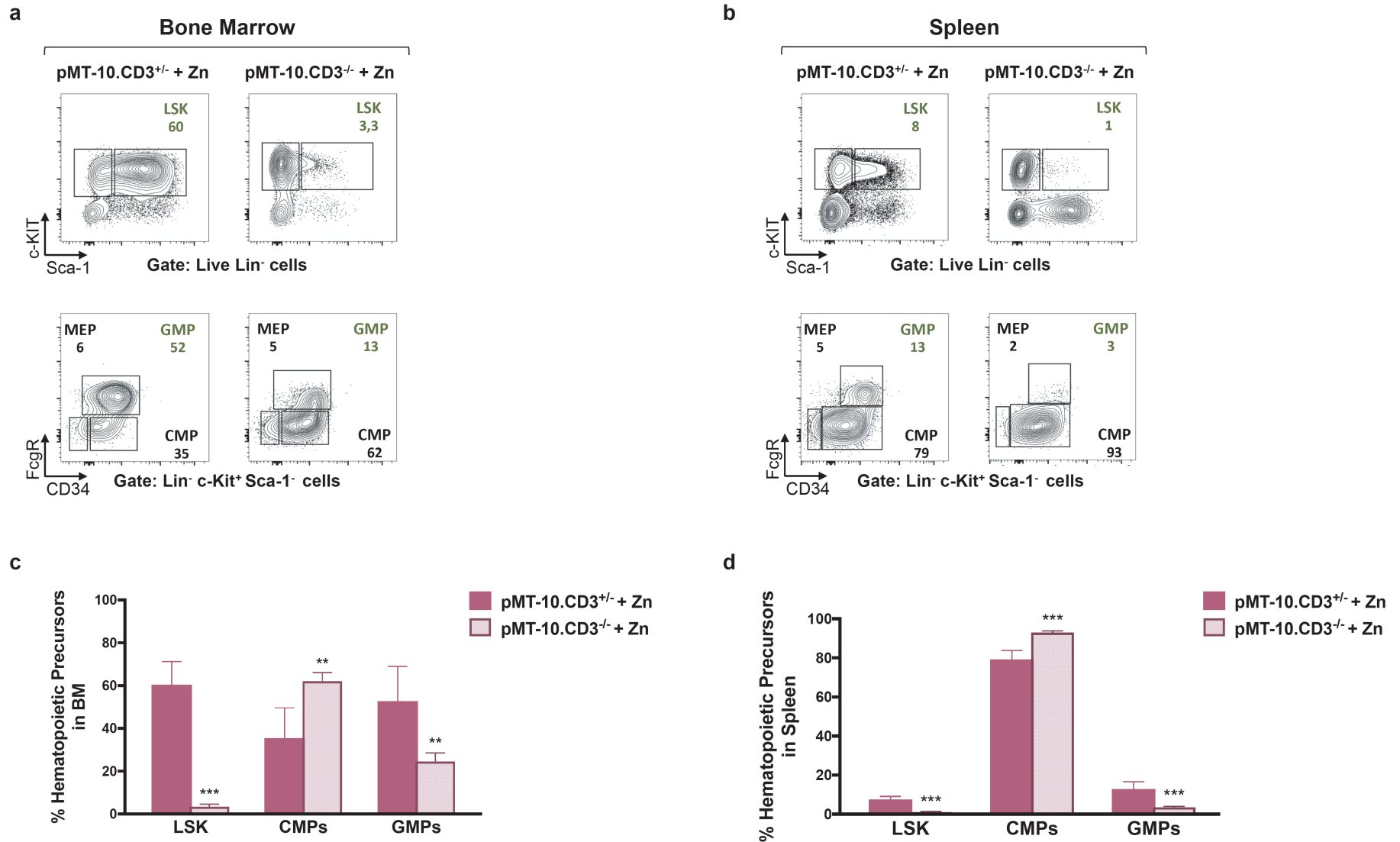

Fig. 3.4, Cardoso *et al*

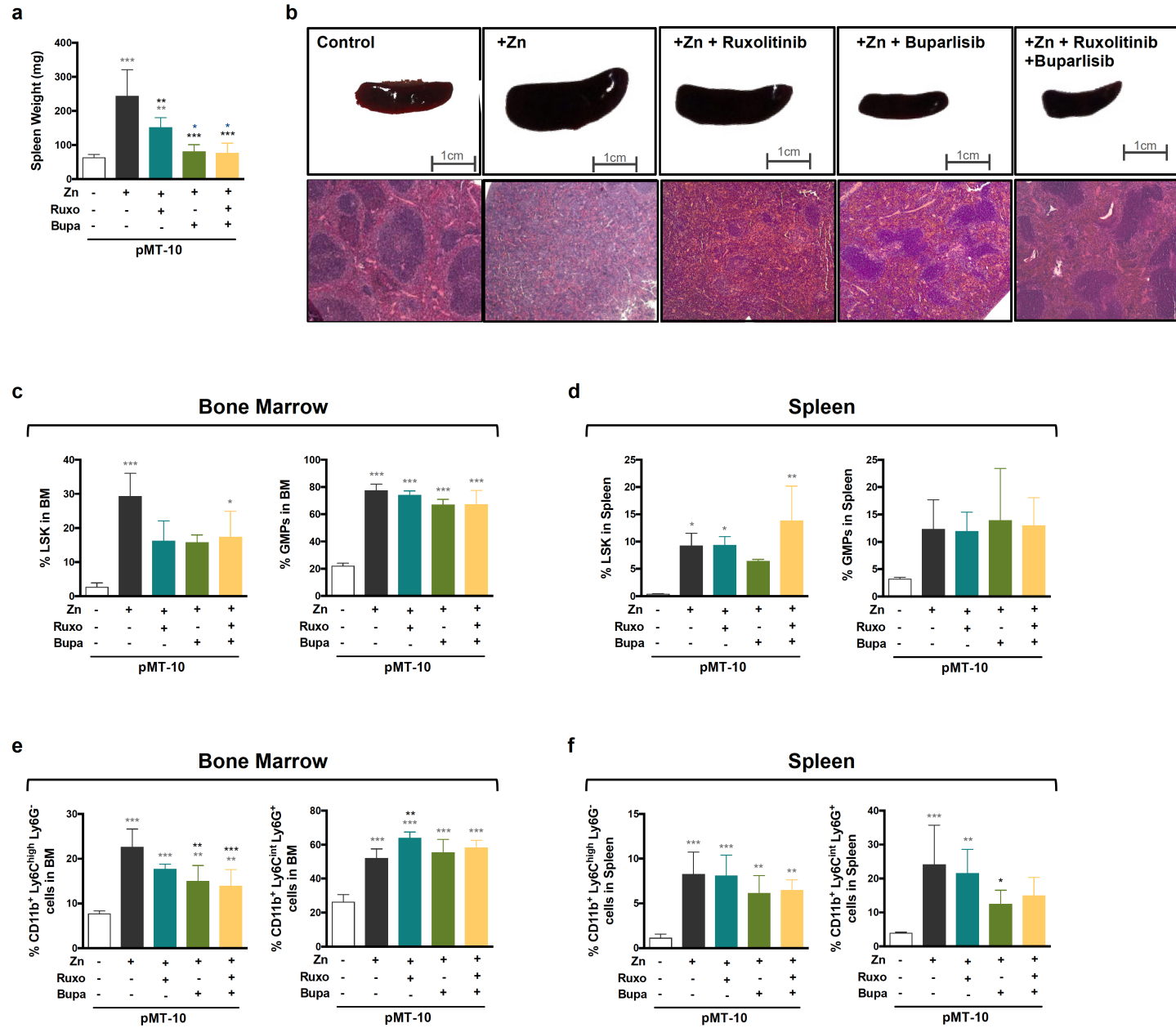**b**

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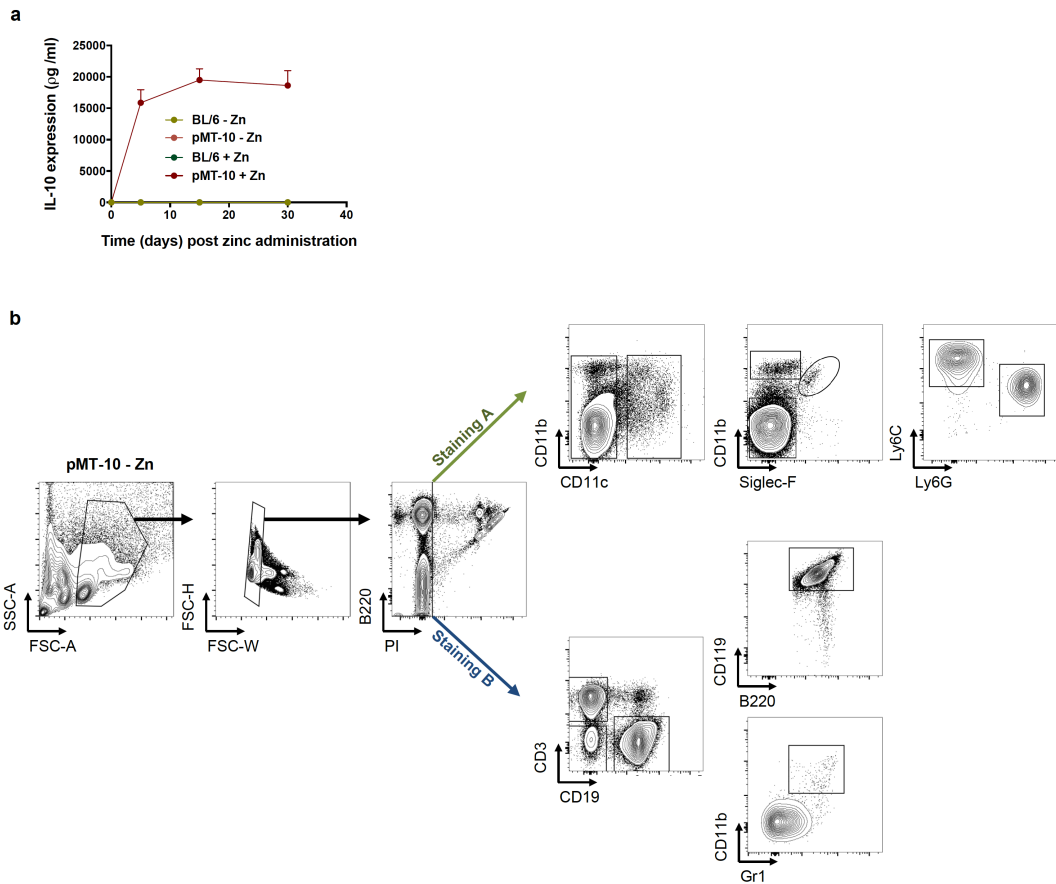
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Spleen



**d**   **e**   **f**   **g**

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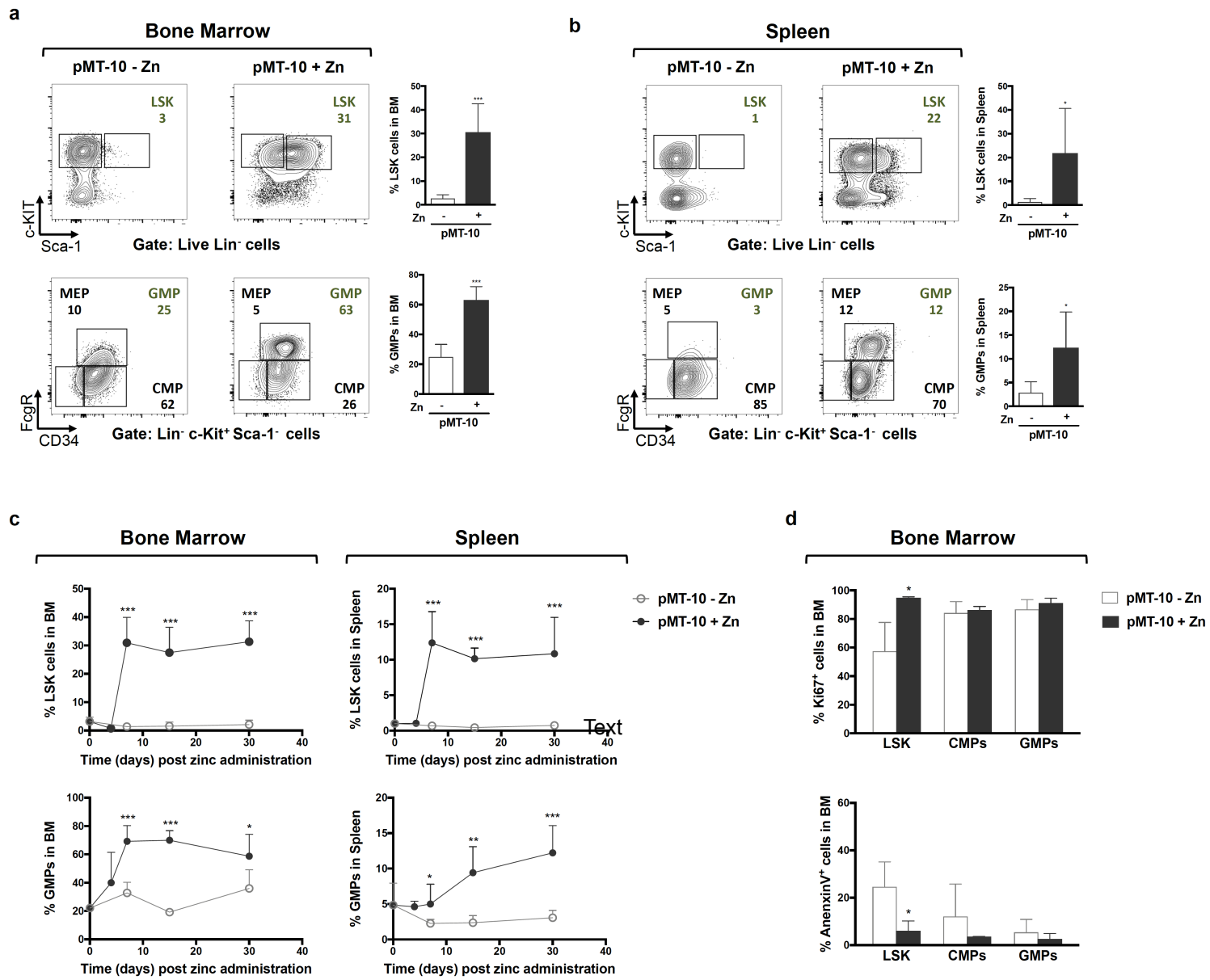
Fig. 3.8, Cardoso *et al*

Supplementary Material


Supplementary Fig 3.1. Kinetics of IL-10 over-expression in the serum at different time points post Zn administration. (a) BL/6 and pMT-10 mice were fed with normal or Zn-enriched water and at the indicated time points blood was harvested and the amount of IL-10 in serum measured by immunoassay. **(b)** Shown is the gating strategy for lymphoid and myeloid cells analysis, in pMT-10 mice.

a

b
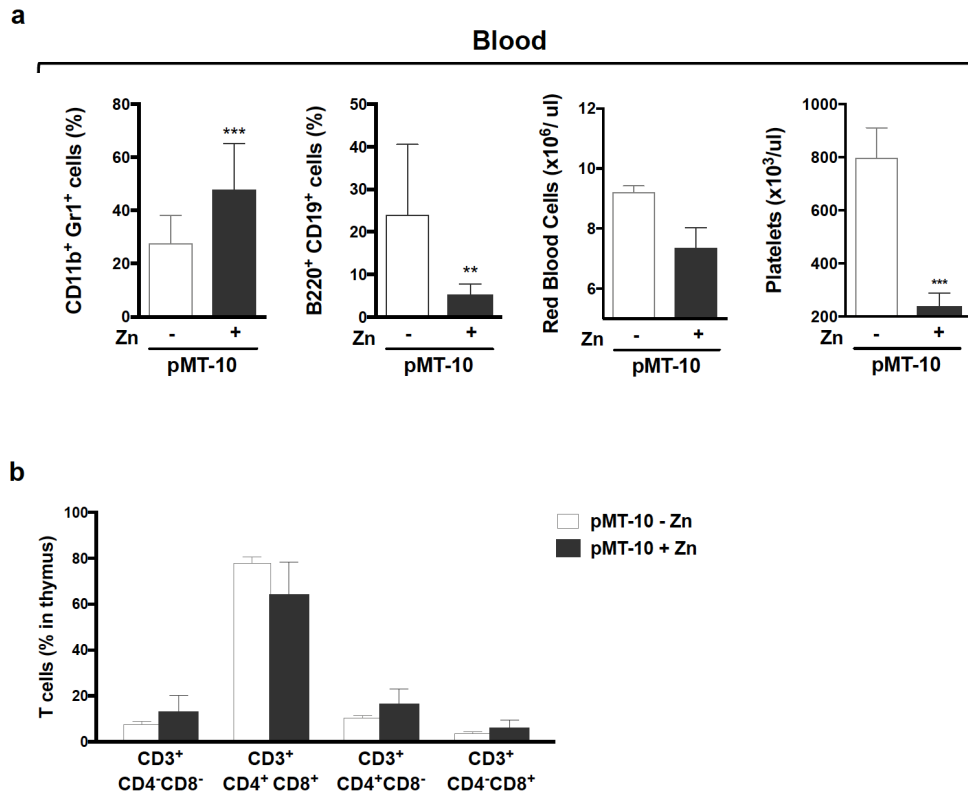
Supplementary Fig 3.2. Peripheral blood analysis showed a marked reduction of red blood cells and platelets in mice expressing IL-10. IL-10 over-expression in pMT-10 mice was induced for 30 days. **(a)** At day 30, percentage of CD11b^{+} Gr1^{+} and B cells, and number of red blood cells and platelets in the peripheral blood of pMT-10-Zn and pMT-10+Zn was recorded. **(b)** Numbers of T cells in the thymus, at day 30 0f IL-10 over-expression, based on the cell surface expression of CD3, CD4 and CD8. Data from one representative experiment with Mean±SD for 6 independent mice. Data were analyzed with student's t test **p<0.01; ***p<0.001.
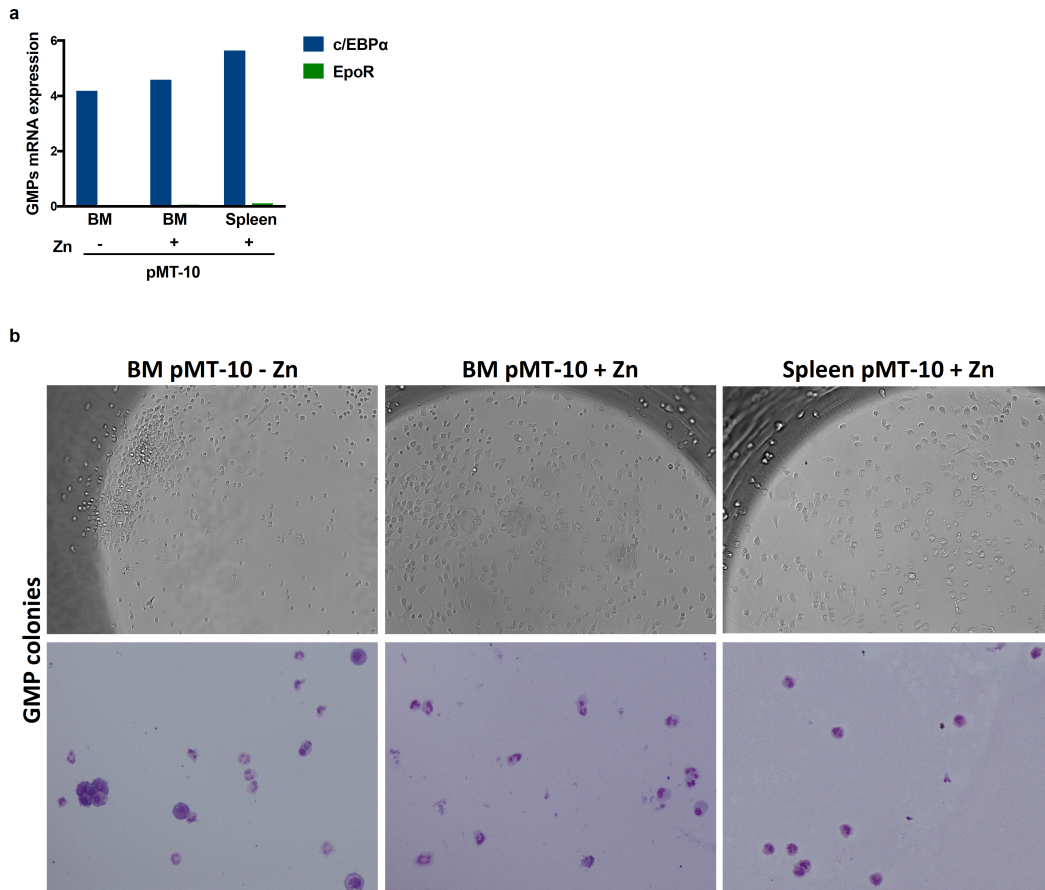
Supplementary Fig 3.3. pMT-10 mice over-expressing IL-10 develop extramedullary myelopoiesis. IL-10 over-expression in pMT-10 mice was induced for 30 days. At day 30, BM and splenic GMPs from pMT-10-Zn and pMT-10+Zn were sorted based on the expression of IL-7Rα, c-Kit, Sca-1, FcγR and CD34. **(a)** Transcriptional analysis of sorted GMPs. **(b)** Representative photos of GMPs colonies, from all groups, after 6 days of differentiation and respective May-Grünwald staining.
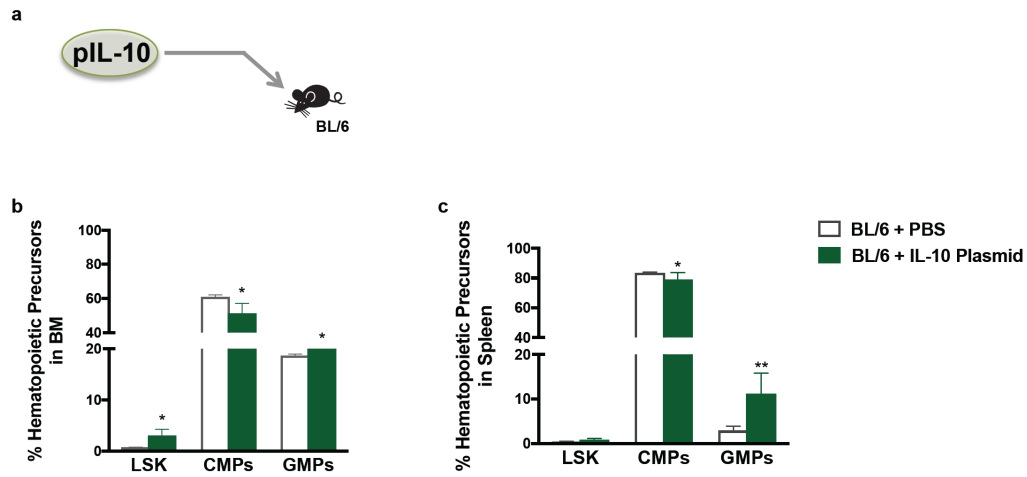
Supplementary Fig 3.4. The IL-10-driven myelopoiesis is phenocopied in a different induction model. (a) An IL-10-expressing plasmid was injected into BL/6 mice, in PBS. Vehicle control was PBS alone. James di Santo and Yan Li provided the plasmid used and helped with animal handling. After 7 days, leucocyte populations were assessed using the same cell surface markers as before in the **(b)** BM and **(c)** spleen. Data from one experiment with Mean±SD, for 3-6 independent mice. Data were analyzed with student's t test *p<0.05; **p<0.01.
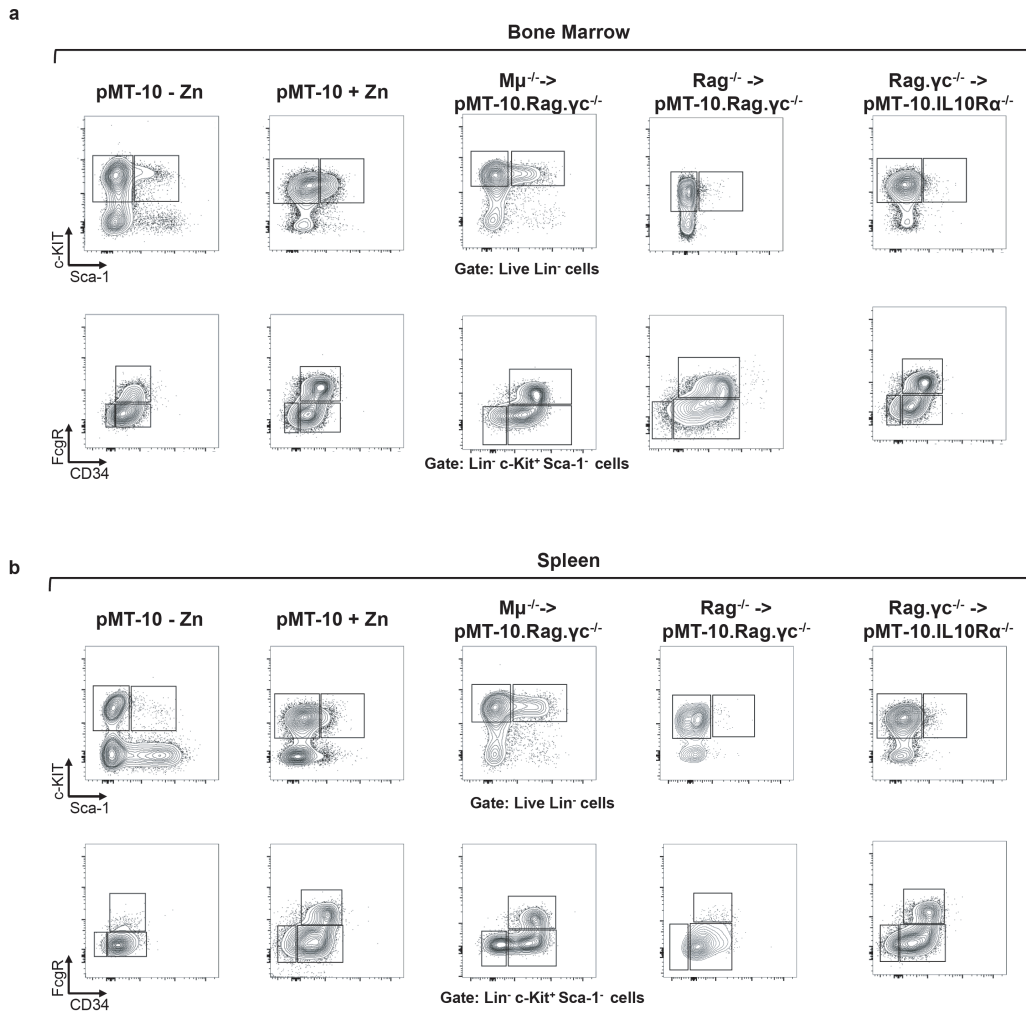
**Supplementary Fig 3.5. B, NK and ILCs play a redundant role in IL-10 driven myelopoiesis.** Transplantation assays were performed by injecting 4×10^6 T cell-depleted $M\mu^{-/-}$ BM cells or total BM of $Rag^{-/-}$ mice into pMT-10.Rag.γc-/- mice. As controls we used pMT-10 fed with normal or Zn-enriched water, and irradiated pMT-10.IL-10R$\alpha^{-/-}$ mice reconstituted with Rag.γc$^{-/-}$ BM cells. 5 weeks after irradiation and reconstitution, Zn was administered to the animals for two weeks. Representative plots of **(a)** BM and **(b)** spleen LSK cells and myeloid precursor populations of each group.
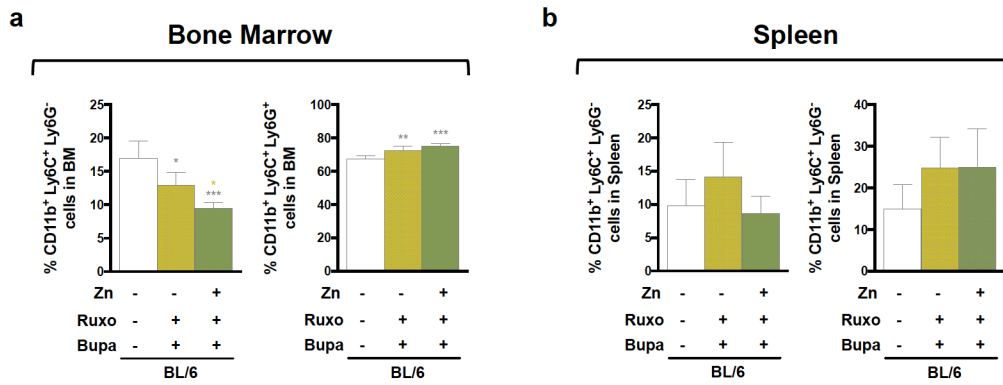
Supplementary Fig 3.6. JAK2 and PI3K inhibitors administration impact myeloid subsets in normal mice. Normal or Zn-enriched water was administered to BL/6 mice for 30 days. At day 30, inhibitors or vehicle were administrated by oral gavage in parallel with Zn in the drinking water. At day 37, **(a)** BM progenitor population were assessed using the same cell surface markers as before. Myeloid mature subsets in the **(b)** BM and **(c)** spleen. mature subsets in the BM and spleen. Data from one representative experiments, with Mean±SD for 5-6 independent mice. Data were analyzed with one-way ANOVA $*p<0.05$; $**p<0.01$; $***p<0.001$.
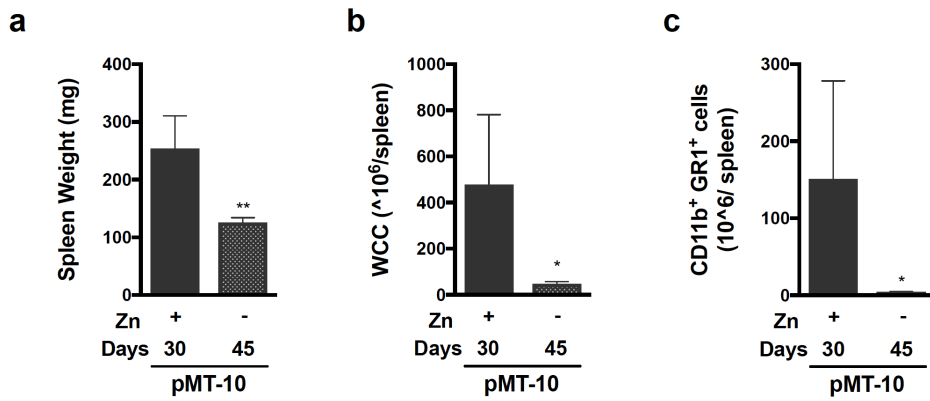
Supplementary Fig 3.7. IL-10 withdrawal restores normal hematopoiesis. At day 45, 15 days after the end of Zn administration, spleens from pMT-10 previously fed with normal or Zn enriched water were harvested, and spleen **(a)** weight, **(b)** total number of leukocytes and **(c)** number of monocytes/ neutrophils (CD11b⁺ Gr1⁺) assessed. Each bar represents the Mean±SD for 6 independent mice, in 2 independent experiments. Data were analyzed with student's t-test *$p<0.05$; **$p<0.01$.

CHAPTER IV

Discussion

General Discussion

Interleukin (IL)-10 was described over two decades ago as a factor produced by T helper (h) 2 cells, that inhibited interferon (IFN)-γ production by Th1 cells [1]. However, it later became evident that IL-10 is, in fact, produced by many cell types [2]. The expression of IL-10 is tightly regulated, and this cytokine exerts an anti-inflammatory effect in many cells, thus playing a prominent role in the regulation of the immune response. Furthermore, because IL-10 is deregulated in the context of several diseases, its biological activity has been targeted for therapy [3, 4].

The main goal of this work was to unveil novel aspects on the role of IL-10 in the homeostasis of the hematopoietic system. Although IL-10 is amply studied in the context of disease, as infection, cancer and autoimmunity, its impact in homeostatic conditions is largely unknown. This is however important, especially considering the potential therapeutic applications of IL-10. Furthermore, this knowledge is expected to strengthen the connection between all the players during the inflammatory response and hematopoietic output.

In this Thesis, we proposed to study the role of IL-10 in experimental-induced disease conditions, while also analyzing its impact alone on organism homeostasis. In short, we attempted to provide evidence that would place IL-10 as non-redundant modulator of the hematopoietic process. To address this, two main goals were defined: i) to understand how an anti-inflammatory scenario, provided by transient IL-10 over-expression, could modulate cell maturation profiles in response to gut induced pathology; and ii) to explore the effects of IL-10 in medullary hematopoiesis.

Most research focused on investigating the role of IL-10 in cell-mediated and humoral immunity has involved the administration of neutralizing antibodies or of large amounts of recombinant IL-10 to experimental animals or cultured cells. While these studies are helpful in outlining IL-10 functions, it is difficult to regulate cytokine dose and timing by these means. Thus, several genetic tools were generated throughout the years to dissect the precise cellular consequences of loss or over-expression of IL-10 expression and its signaling [5-10]. The first part of this work involved the characterization of a novel mouse model for inducible IL-10 over-expression. The strain was generated using a construct containing IL-10 cDNA under the control of the inducible sheep metalloprotein promoter. This promoter is activated in the

presence of 50mM of Zinc (Zn) in the organism, administered in the drinking water, with IL-10 being expressed by CD45 TER119 cells from the small intestine (SI), skin and, to a less extent, bone marrow (BM). Thus, the pMT-10 mouse model allows for timely controlled IL-10 over-expression in specific anatomic locations, in addition to a significant increase of the levels of this cytokine in the serum. Importantly, the pMT-10 mouse model is not dependent on cell activation as the previous reported models [5-8], providing an unbiased tool to explore the role of IL-10 in the organism.

IL-10 is an essential molecular effector of intestinal regulation. Thus, the study of the mechanisms that promote IL-10-mediated protection is crucial to unveil novel therapeutic targets for Intestinal bowel disease (IBD). Our approach, as described in Chapter II, consisted in using a well-described model of dextran sulfate sodium (DSS)-induced colitis [11]. pMT-10 mice pre-conditioned with IL-10 for 8 days before DSS administration showed a milder colitic phenotype. This protection was partially enhanced by the increased concentration of Zn in the organism of these animals, possibly due to a reduction in the inflammatory profile of Ly6C+ cell subset. The fact that we detected increased transcription of the IL-10 transgene in the BM and elevated levels of seric IL-10 led us to hypothesize that pre-exposure to IL-10 might induce long-lasting transcriptional changes in circulating monocytes, for example through epigenetic imprinting. If this were the case, re-educating these cells to gain long-lasting tolerance to DSS-induced colitis would become an attractive possibility. However, this did not occur, as the protective effects of IL-10 were not long lasting, implying that IL-10 presence at the time of insult is necessary to ameliorate colitis.

Nonetheless, dissecting the properties and functions of the Ly6C+ cell subset that encounter an IL-10 environment in the gut, could bring additional information on how this population is delaying disease progression, revealing new molecules for future targeting. Our results show that IL-10 impacts the inflammatory profile of the Ly6C+ cell subset in the *lamina propria* of pMT-10 mice. In the future, a RNA Seq analysis of the Ly6C+ cell population obtained from the different environments (control, IL-10-enriched or Zn–enriched) may be important to reveal the on-going specific molecular alterations and their functional impact on disease progression. Furthermore, it will be of interest to combine the RNA *Seq* with ChIP *Seq* analysis, to evaluate the occurrence of epigenetic modifications, and how they may modulate the transcriptional profile of the Ly6C+ cell subset during IL-10 over-expression.

In all, this part of the work offered a novel mouse model of induced IL-10 over-expression and highlighted new possibilities for the mechanism that allows IL-10 to control intestinal inflammation.

The second part of this Thesis focused on hematopoietic regulation by IL-10. The signals that regulate cell differentiation, proliferation and survival are activated by molecular programs that determine the specification of the precursors along the different lineages [12-16]. The influence of several cytokines, such as IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-7, in hematopoiesis has been well reported over the years [17-22]. However, a role for IL-10 in this process remained unknown.

During infections, the hematopoietic output may be dramatically altered, as steady-state hematopoiesis switches to emergency hematopoiesis by triggering a unique hematopoietic response program that is aimed at increasing myeloid cell output to meet the organism demand [23, 24]. Though the molecular mechanisms underlying regulation of emergency hematopoiesis are not fully understood, recent evidence suggests that the expression of various mediators, notably cytokines, have a non-redundant role on this switch in cell differentiation [25]. Understanding whether IL-10 may modulate hematopoietic differentiation is of unquestionable importance, considering the possible applications of IL-10 modulation in inflammatory diseases [4].

In this study, we provide evidence that IL-10 over-expression impacts hematopoiesis, by promoting a deviation of the hematopoietic process towards myelopoiesis, a process that parallels emergency hematopoiesis. Indeed, we show that high levels of IL-10 lead to an increase of Lin$^-$ SCA-1$^+$ cKIT$^+$ (LSK) and granulocytic-macrophage progenitors (GMPs) population in the BM, along with extramedullary accumulation of the same subsets in the spleen. The expansion of LSK cells and GMPs started in the BM soon after IL-10 induction in pMT-10 mice, while its splenic counterparts only became significantly altered later on. These observations suggest that IL-10 modulates medullary cell differentiation leading to an extramedullary hematopoiesis upon the egress of progenitors from the BM to the spleen. The cause of this cell migration from the BM to the spleen may reside on an altered BM niche. It is conceivable that IL-10 impacts not only hematopoietic cell differentiation, but also niche homeostasis, promoting signals for cell migration to organs able to support hematopoiesis. On the other hand, the hematopoietic progenitors may be successful in colonizing the spleen

simply because there are more progenitors in circulation. All these hypothesis may be coupled with the fact that the splenic niche is also affected, expressing signals for cell recruitment and colonization of the organ. It will be important, in the future, to address this various possibilities and to define in a more detailed way the molecular cues underlying the IL-10-driven extramedullary myelopoiesis.

We describe an enhanced LSK proliferation and survival, both in the BM and spleen, correlating with a sustained expansion of the GMPs during IL-10 over-expression. An outstanding question relates to the identification of the populations that, within LSK cells, are altered by IL-10. Preliminary data show that pMT-10 mice over-expressing IL-10 present a higher expression of CD48 in BM LSK CD150+ cells than that observed in controls (data not shown). To further investigate the authenticity of the expanded BM LSK cell subsets in pMT-10 mice, a more detailed FACS analysis, comprising IL-7Rα, Sca-1, c-Kit, FcγR, CD34, CD48, CD150 and Flt3 altogether is currently on going. The combination of these markers will allow us to identify long-term (LT)-hematopoietic stem cells (HSCs), short-term (ST)-HSCs, lymphoid primed progenitors (LMPPs) and multipotent potent progenitors (MPPs) enclosed in the LSK compartment [26, 27]. Differentiation potential, through *in vitro assays,* will provide further evidence that the expanded LSK subsets correspond to *bona fide* BM progenitors. Furthermore, single cell RNA *Seq* on LSK cells as well as conventional RNA analysis would be of interest to check for their transcriptional identity. However, the final evidence that those cells are hematopoietic progenitors would reside on their ability to successfully reconstitute hematopoietic lineages in lethally-irradiated recipient mice. All these experiments are currently on going.

Another interesting question raised by this study is how IL-10 promotes a bias for myeloid differentiation. One possibility is that IL-10 might induce transcriptional changes in BM progenitors, for example through epigenetic imprinting that could modulate cell fate. Therefore, the RNA *Seq* analysis of the BM progenitor populations will also be shed light into lineage commitment molecular alterations and their functional impact during IL-10 conditioned differentiation.

Using BM transfer models, we have further showed that IL-10 modulates hematopoiesis through a direct and indirect mechanism. Our findings suggest that IL-10 acts at the BM level, initiating an expansion of LSK cells. However, IL-10 requires the action of other intermediate

cell subsets for LSK expansion, as in the absence of T cells the phenotype is lost. We thus hypothesize that, in response to IL-10, another molecular mediator is required to instruct the downstream T cell response. Importantly, our experiments with reconstituted pMT-10.IL-10Rα-/- mice show that T cells are not responding directly to IL-10, hence the need of an intermediate signal. Thus, RNA *Seq* analysis of the different T cell subsets would be of interest, as it could provide further evidence on the upstream and downstream factors involved on IL-10-driven myelopoiesis.

Several reports demonstrated that myeloid differentiation is regulated by key growth factors, such as GM-CSF. GM-CSF signaling is critical for monocyte differentiation and survival [20, 22, 28], and an association between GM-CSF receptor (GM-CSFR) hypersensitive and myeloproliferation has been described [29-31]. Combining this information with our results, we hypothesized a role for GM-CSF in our model of IL-10-driven myeloexpansion. Serum analysis of induced pMT-10 mice showed no alteration in the GM-CSF expression when compared to control pMT-10 mice (Annex I, Supplementary Fig. 4.1). Of note, we also failed to detect differences in other most probable candidates, namely cytokines that are known to regulate myelopoiesis, as M-CSF, IL-3 and G-CSF (Annex I, Supplementary Fig. 4.1). Still, it is conceivable that IL-10-induced cytokine deregulation operates in specific anatomical locations and is, thus, not reflected in the serum. To address this problem, we will evaluate the transcriptional profile of different cell subsets from different organs, as the BM, SI and spleen. Administration of anti-GM-CSF to pMT-10 mice over-expressing IL-10 had no effect on the characteristic myeloexpansion (Annex I, Supplementary Fig. 4.2).

Interestingly, the phenotype revealed upon IL-10 over-expression mainly parallels that of myeloproliferative neoplasms (MPNs) in animal models and humans [32-34]. MPNs are a heterogeneous group of clonal diseases characterized by the excessive and chronic production of mature cells from one or several of the myeloid lineages [32]. Due to the relevance of Janus Kinase (JAK) 2 mutations for the etiology of MPNs, several JAK inhibitors are used in the clinics, with very positive results. Most interestingly, JAK inhibitors also work in individuals who do not present (known) JAK2 mutations, thus suggesting that hyper-activation of JAK2 may be a common factor driving myeloproliferation.

Administration of JAK2 (Ruxolitinib (Ruxo)) and/or phosphoinositide-3-kinase (PI3K) (Buparlisib (Bupa)) inhibitors to pMT-10 mice over-expressing IL-10 showed small only effects.

Despite the fact that PI3K inhibitor was able to block BM LSK proliferation/ differentiation in pMT-10 mice over-expressing IL-10, we must consider

that these inhibitors act by impairing actively proliferating cells. Thus, the effects of the PI3K inhibitor might not be due not the fact that IL-10 is signaling through this pathways, but rather because the progenitors have an enhanced proliferation profile. Since our results suggest that pMT-10 mice over-expressing IL-10 develop several alterations that parallel MPNs, it would be of interest to in the future expand our observations by evaluating a possible association of genetic variants in *IL10* or in genes involved in the IL-10 signaling and MPNs in human patients.

IL-10 expression is one of the most important mechanisms evolved by many immune cells to counteract damage driven by excessive inflammation. Thus, downregulation of IL-10 expression may lead to the development of severe forms of immunopathologies through a sustained or enhanced inflammatory response as an upregulation can lead to chronicity. Considering the poor outcomes of the past IL-10 clinical trials for the treatment of autoimmune disorders, it has become clear that a more detailed appreciation on how the source and kinetics of IL-10 expression modulates an effective immune response and interaction with different cell subsets is in order.

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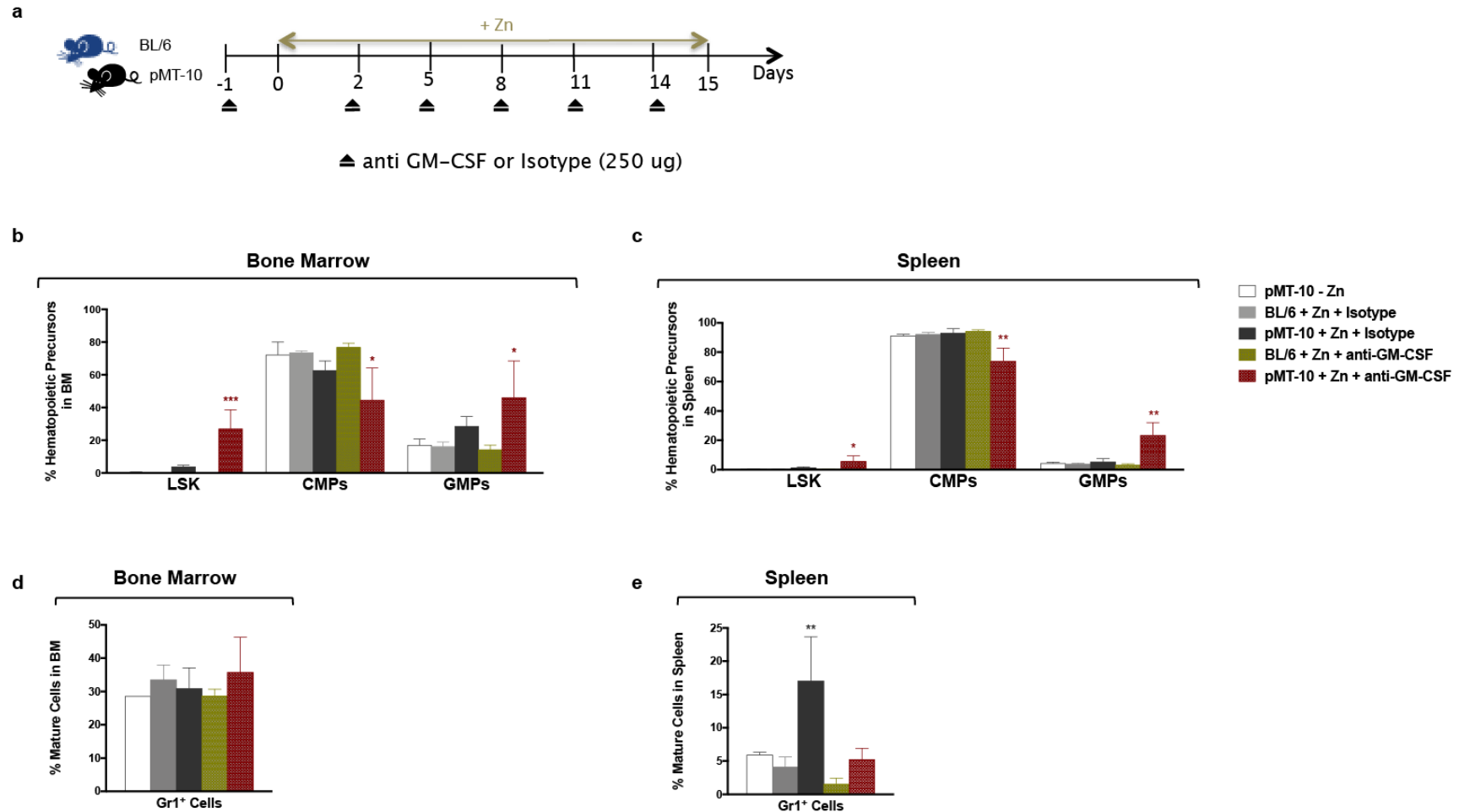
Appendix

Supplementary Data

Appendix I - Chapter IV: supplementary data
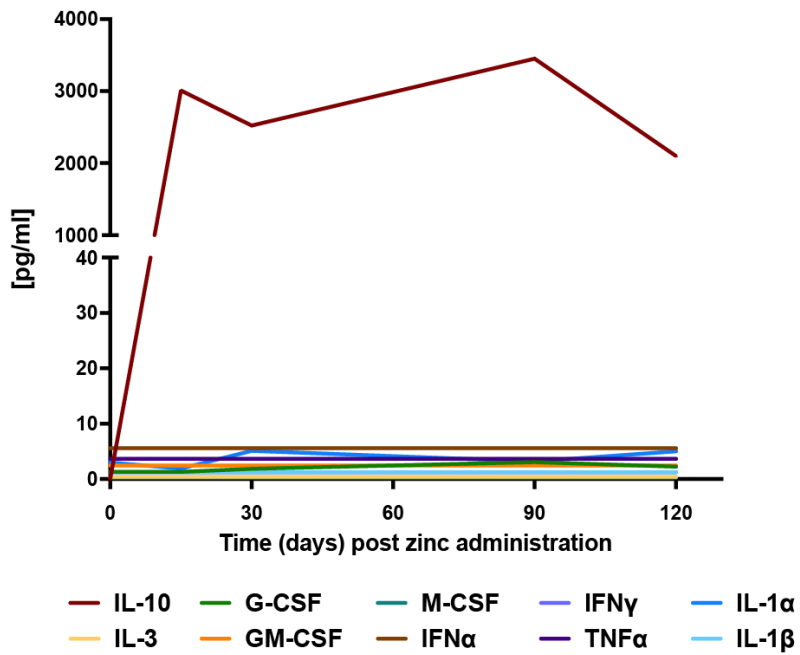
Supplementary Fig 4.1. Cytokine profile in induced pMT-10 mice. (a) Serum was collected at day 15, 30, 90 and 120 after the beginning of zinc administration from induced animals.

Supplementary Fig 4.2. GM-CSF is not a key factor in IL-10 driven myeloexpansion. (a) Induced pMT-10 mice were injected every two days for 3 weeks with either anti-GM-CSF mAb or isotype control, and compared with non-induced mice. **(b-e)** Progenitor populations in BM and spleen were assessed at day 15 using the same cell surface markers as before. **(b-e)** Data from one representative experiment with Mean±SD, for 3-6 independent mice per group. Data were analyzed with one-way ANOVA*$p<0.05$; **$p<0.01$; **$p<0.001$.