Understanding the role of extrinsic regulators in normal and malignant haematopoiesis

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted or is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

Abstract

Name: Miriam Belmonte

Thesis title: Understanding the role of extrinsic regulators in normal and malignant haematopoiesis

Numerous studies have highlighted the central role that extrinsic regulators play in both normal and malignant haematopoiesis. Recent evidence suggests that variations in key molecules can directly modulate haematopoietic stem cell (HSC) fate, implicating non-cell intrinsic mechanisms for both maintaining homeostasis and for driving disease development. This thesis spans both normal and malignant haematopoiesis, assessing the impact of extrinsic regulators on HSC fate and explores their role in driving the evolution of clonal haematological malignancies.

First, I explored the impact of extrinsic regulators on highly purified single HSCs and found that minimising cytokine signalling could sustain a hibernation state in HSCs with the retention of functional properties both *in vitro* and *in vivo.* This work established the principle that core functions of HSCs could be maintained in the absence of the bone marrow microenvironment and allowed the identification of key factors dispensable for HSC function (Chapter 3).

Next, to understand the role of extrinsic regulators in disease evolution I shifted focus to characterising the cytokine microenvironment in patients with preleukaemic HSC-derived disorders known as the myeloproliferative neoplasms (MPNs). This work identified key regulators of the preleukaemic state, including two potential biomarkers of disease evolution, and additional molecules that associated with advanced disease (Chapter 4).

This correlation of cytokine levels with specific disease subtypes and disease severity led to the further exploration of one of these molecules in the context of MPNs. The final chapter of my thesis therefore focused on the functional characterisation of IP-10, whose levels correlated with a more severe disease subtype and associated with JAK2 and TET2 mutational status in both patients and mouse models (Chapter 5).

Together, these findings highlight the importance of the extrinsic regulators and the haematopoietic microenvironment in influencing cellular outcomes in both the normal and malignant setting and underscores the experimental and clinical potential of modulating extrinsic regulators of HSCs.

Preface

Chapter 1

Some of the figures were designed and produced by Mairi Shepherd, a fellow PhD student in the Kent Lab.

Chapter 3

All work was carried out under the supervision of David Kent and Elisa Laurenti. Caroline Oedekoven and I carried out animal and human sample preparation as well as cell culture work. Caroline Oedekoven and I carried out all animal experiments after receiving training from David Kent, Tina Hamilton and Dean Pask, except for the xenotransplantation experiments which were carried out by Nicole Mende and Emily Calderbank. Adam Wilkinson (Stanford University) provided us with technical advice regarding the medium composition for the serum-free culture and the process of near-complete medium changes. Nicola Wilson, Winnie Lau and Sonia Nestorowa developed the modified protocol of single-cell RNA sequencing used in this thesis. RNA library preparation was carried out at the Genomics Core facility of the Cambridge Stem Cell Institute. The bioinformatic analysis of the single cell RNA sequencing data was mainly carried out by Fiona Hamey, Daniel Bode, Hugo Bastos and Lila Diamanti, with input from myself, Caroline Oedekoven and David Kent. All mouse single cell sorts were carried out at the Flow Cytometry Core Facility at the Cambridge Institute for Medical Research (CIMR) by Reiner Schulte, Chiara Cossetti, and Gabriela Grondys-Kotarba. All human single cell sorts were carried out at the NIHR Cambridge BRC Cell Phenotyping Hub facility.

Chapter 4

All work was carried out under the supervision of David Kent. Me and Nina Øbro carried out human sample preparation as well as multi-plex ELISA assay. Milliplex plates were run at the MRC Laboratory of Molecular Biology (LMB) with the technical assistance of Helen Jolin and Jillian Barlow. The bioinformatic analysis and integration of clinical and genomic data was carried out by Jacob Grinfeld with input from myself, Nina Øbro, and David Kent.

Chapter 5

All work was carried out under the supervision of David Kent. I led and carried out all animal experiments, assisted by Tina Hamilton and Dean Pask for peripheral blood sample collection and by James Che and Caroline Oedekoven for HSC transplantations. Me and Nina Øbro carried out mouse sample preparation as well as multi-plex ELISA assay. Milliplex plates were run at the MRC Laboratory of Molecular Biology (LMB) with the technical assistance of Helen Jolin and Jillian Barlow.

All single cell sorts were carried out at the Flow Cytometry Core Facility at the Cambridge Research UK (CRUK) by Richard Grenfell and Mateusz Strzelecki, and at the Flow Cytometry Core Facility at the Cambridge Institute for Medical Research (CIMR) by Reiner Schulte, Chiara Cossetti, and Gabriela Grondys-Kotarba.

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I am grateful for the amazing lab mates I spent my every-day life with over the past 4 years. Thank you for the stimulating conversations, for keeping the snack drawer always full, and for being great friends rather than just colleagues. Thanks especially to Nina Øbro, Mairi Shepherd, James Che, Craig McDonald, and Daniel Bode.

Professionally, my PhD would have not been possible without the help of the CIMR Flow Core Facility. Thank you, Reiner, Chiara, and Gabi, for the countless number of sorts and for your patience and kindness. None of the work of this thesis would have been possible without you guys.

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Finally, my deepest gratitude goes to my sister and to my parents. None of this would exist without the love and constant support they have given me throughout my whole life.

Table of Contents

Table of Figures

List of Tables

Abbreviations

7AAD; 7-Aminoactinomycid D
ANGPT1; Angiopoietin-1
ALL; Acute lymphoblastic leukaemia
Allo-HSCT; Allogeneic haematopoietic stem cell transplant
AML; Acute myeloid leukaemia
APC; Allophycocyanin
ARCH; Age-related clonal haematopoiesis
BM; Bone marrow
BV; Brilliant violet
BSA; Bovine serum albumin
CALR; Calreticulin
CAMPT; Congenital amegakaryocytic thrombocytopenia
CB; Cord blood
CBL; Casitas B-cell lymphoma
CCL3; C-C ligand 3
cDNA; Complementary deoxyribonucleic acid
CFC; Colony forming cell
CFU; Colony forming unit
CMML; Chronic myelomonocytic leukaemia
CML; Chronic myeloid leukaemia
DL1; Delta-1
DNMT3A; DNA methyltransferase 3A
E.; Mouse embryonic day
ECM; Extracellular matrix
EGF; Epidermal growth factor
EPCR; Endothelial protein C receptor
ER; Endoplasmic reticulum
ET; Essential thrombocythaemia
FACS; Fluorescence-activated Cell Sorting
FBS; Foetal bovine serum
FGF1; Fibroblast growth factor 1
FL; Foetal liver
Flk2; Foetal liver kinase 2
FLT3; fms-like tyrosine kinase 3
FLT3-L; FLT-3 ligand
G-CSF; Granulocyte colony-stimulating factor
GSCA; Gene set enrichment analysis
(GVHD); Graft-versus-host disease
Hb; Haemoglobin
HCT; Haematocrit
HGB; Haemoglobin
HGF; Hepatocyte growth factor
HSC; Haematopoietic Stem Cell
HSPC; Haematopoietic Stem cell and progenitors
IGF2; Insulin growth factor 2
IGFBP2; Insulin growth factor-binding protein 2
IFN; Interferon
IL; Interleukin
IP-10; Interferon gamma-induced protein 10
IRF2; Interferon regulatory factor 2
JAK2; Janus Kinase 2
KI; Knock-in
KO; Knock-out
KL; Kit-ligand
LDA; Limiting Dilution Assay
LepR; Leptin receptor
Lin; Lineage
LPS; Lipopolysaccharides
LSK; Lineage-Sca1+c-kit+
LT-HSCs; Long-term HSCs
MDS; Myelodysplastic syndrome
MF; Myelofibrosis
MGF; Mast cell growth factor
MolO; Molecular Overlapping
MPN; Myeloproliferative neoplasm
MSC; mesenchymal stromal cell
NGF; Nerve growth factor
NSG; NOD scid gamma
OPN; Osteopontin
PAMP; Pathogen-associated molecular pattern
PB; Peripheral blood
PCA; Principal component analysis
Ph; Philadelphia chromosomal translocation
PKB; Protein kinase B
pMF; Primary myelofibrosis
PPR; pattern recognition receptor
PV; Polycythaemia vera
PVA; Polyvinyl alcohol

RA; Receptor antagonist
RAR-γ; Retinoic acid receptor-gamma
RCC; Renal cell carcinoma
Rb; Retinoblastoma
RBC; Red blood cell
T-ALL; T-cell acute lymphoblastic leukaemia
SCF; Stem cell factor
scRNA-seq; Single-cell RNA sequencing
Sec; Sinusoidal endothelial cells
SEM; Standard error of the mean
SF; Steel factor
sIL2-Rα; Soluble IL-2 receptor alpha
sMF; Secondary myelofibrosis
SOCs; Suppressors of cytokine signalling
SR1; StemRegenin 1
Stem cell antigen-1; Sca-1
STAT; Signal transducers and activators of transcription
TET2; Ten-Eleven Translocation 2
TLR; Toll-like receptor
TPO; Thrombopoietin
TGF-β; Transforming growth factor beta
UMAP; Uniform Manifold Approximation and Projection
VEGF; Vascular endothelial growth factor
WT; Wild type

1 Introduction

1.1 Extrinsic regulators of haematopoietic stem cells and normal haematopoiesis

1.1.1 Haematopoiesis and haematopoietic stem cells

The blood system is a highly regenerative tissue and it is responsible for the daily production of over one trillion (10^{12}) blood cells and for sustaining life-long haematopoisis[1]. Haematopoiesis is the process of mature blood cell production which takes place in the adult bone marrow (BM) and it is organised as a hierarchical system[2]. Haematopoietic stem cells (HSCs) are thought to reside at the apex of the hierarchy and possess the unique capacity to self-renew, perpetuating themselves as an undifferentiated population, and to differentiate, generating the correct numbers and types of mature cell progeny to perform the necessary functions of the blood system.

Through a gradual fate restriction process, HSCs give rise to progenitor cell intermediates that eventually produce myeloid and lymphoid blood cell types (or "lineages"):

- Myeloid cells, which include macrophages, granulocytes (neutrophils, basophils and eosinophils) and some dendritic cells, involved in immunologic response and inflammation; erythrocytes, involved in O_2 delivering to body tissues and circulation; and platelets or thrombocytes, involved in blood coagulation and tissue repair;
- Lymphoid cells, which include B and T lymphocytes, some dendritic cell and innate lymphoid cells (e.g., natural killer cells), which play a central role in innate and adaptive immune response (Figure 1).

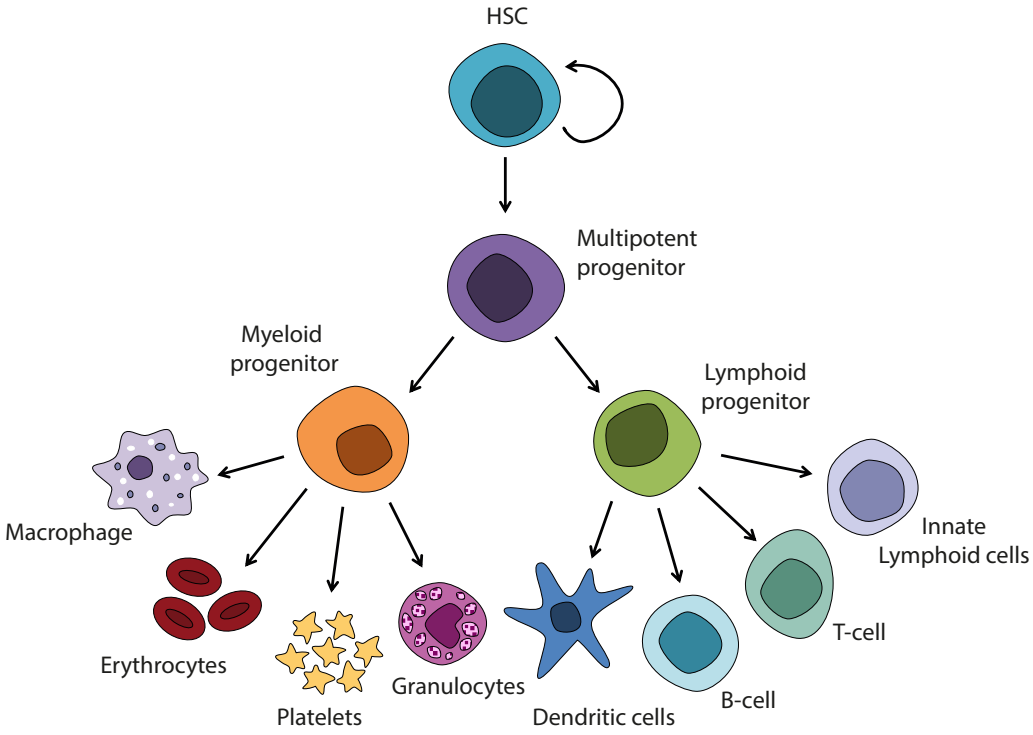


Figure 1. Simplified schematic of the haematopoietic hierarchy.
In normal haematopoietic development, HSCs divide and differentiate, eventually giving rise to the mature myeloid and lymphoid lineages of blood cells. Myeloid progenitors will generate macrophages, erythrocytes, platelets and granulocytes, while the lymphoid branch will give rise to B- and T- cells, dendritic cells and innate lymphoid cells. Illustration by Mairi Shepherd.

A precise balance between HSC self-renewal and differentiation is critical to sustain blood cell homeostasis, maintaining the HSC pool size while providing more differentiated progenitors. Disruption of this balance can result in severe dysregulation of blood cell production where excessive differentiation or insufficient self-renewal leads to HSC pool exhaustion, while insufficient differentiation or unrestrained self-renewal can cause an accumulation of immature cells and a defect in mature cell production. Such uncontrolled growth can sustain leukemic-progression and lead to the production of defective mature blood cells, at the expense of normal haematopoiesis (Figure 2).

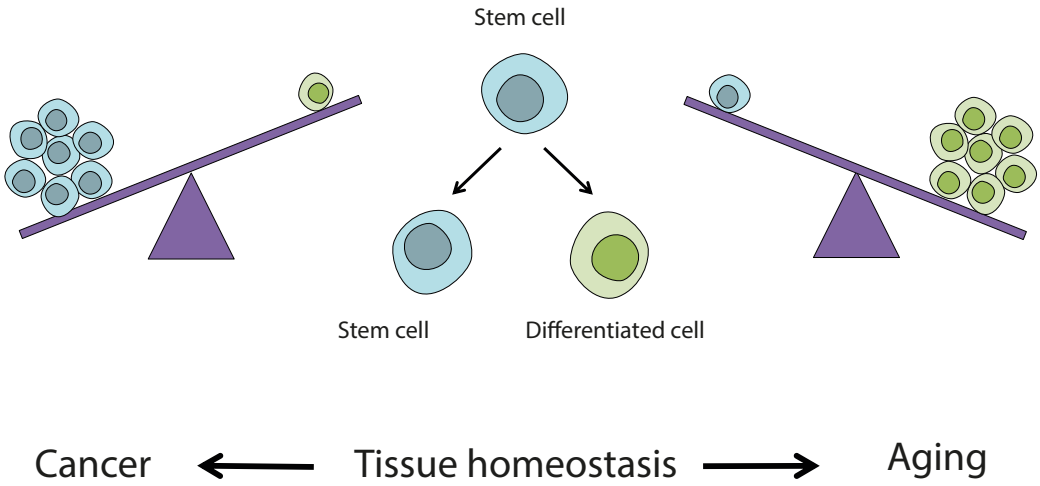


Figure 2. Schematic of HSC properties and consequences of unbalanced haematopoiesis.
HSCs possess the dual ability of self-renewal, giving rise to more equally potent HSCs, and differentiation, generating all mature blood cell types. At the population level, a balance between these two properties must be maintained in order to ensure tissue homeostasis. Increased self-renewal can lead to uncontrolled expansion of the stem cell pool, which, in lack of maturation, can result in haematological malignancies and in more severe cases leukaemia. Excess of differentiation can lead to the exhaustion of the HSC pool. Illustration by Mairi Shepherd.

1.1.2 Isolation of mouse and human HSCs

The definition of the BM as the "seed-tissue" of all blood lineages dates back to 1866[3]. Fifty years later, Alexander Maximov proposed a theory where a common cell could generate the entire tissue, as well as the idea of a micro-environmental niche for these cells within the BM[4]. The first experimental proof that cells could regenerate the entire blood system was obtained in 1951, when Lorenz and colleagues showed that irradiated mice could be rescued from irradiation-lethality and their BM could be re-populated, by injection of marrow or spleen cells from non-irradiated donors[5]. Ten years later, Till and McCulloch formally demonstrated the existence of a single self-replicating common progenitor for all mature cell types, by showing that multi-potent mouse progenitors were able to differentiate and give rise to multi-lineage colonies of single cell origin in the spleen of transplanted recipient mice and importantly were able to be re-transplanted in a new mouse to again give rise to multi-lineage colonies[6]. This was the first formal description of a single cell being able to perform the two main tasks of an HSC: self-renewal, and multi-lineage differentiation.

The capacity of a single HSC to on its own successfully repopulate the marrow of a transplanted recipient was not formally demonstrated until 1996, when the first single cell transplantation experiment showed that $CD34^{-/low}$c-Kit^{+}Stem cell antigen-1 $(Sca\text{-}1)^{+}$Lineage$(Lin)^{-}$ cells could fully re-generate both myeloid and lymphoid compartments in transplanted mice[7]. Since these experiments, the criteria by which HSCs are measured and how they are tracked have changed substantially. The current field standard to demonstrate that a purified HSC is indeed a repopulating cell with durable self-renewal activity is serial transplantation into irradiated mice with long-term multi-lineage reconstitution (>16 weeks) in primary and secondary recipients. To be considered successfully reconstituted, the donor cell contribution must account for at least 1% of the total whole blood count at the later stages of the assay and the multi-lineage contribution is commonly measured by the presence of granulocytes, monocytes, B- and T- cells in the peripheral blood (PB)[8], although erythrocyte and platelet outputs have also been monitored in a number of strategies[9–11].

Multiple groups have since developed different strategies to identify HSCs using their unique characteristics for prospective isolation from the substantially more numerous BM cells. Some research groups sought to isolate them based on size[12,13], density, functional properties[14], such as the ability to efflux Rhodamine 123[15] or Hoechst 33342[16], or the resistance to 5-fluorouracil[17]. Most commonly, researchers have taken advantage of multi-parameter fluorescence-activated cell sorting (FACS) which has greatly facilitated the study of HSCs by identifying cells via their unique cell surface maker expression combinations[18]. The introduction of FACS-based tools such as index-sorting helped to refine sorting strategies and improve HSC purity in both mouse and human. Index-sorting records fluorescence intensity of each immunophenotypic marker for each individual sorted cell. This has been used in combination with single cell functional assays *in vitro*[19] and *in vivo*[20,21] to help improving sort panels based on the output of the assays.

Over the last four decades, a number of combinations have been devised for mouse HSCs including commonly used positive markers such as CD117 (or c-Kit), Sca-1, CD201, (or Endothelial protein C receptor (EPCR)), and CD150, and negative markers such as CD48, CD135 (or Foetal liver kinase 2 (Flk2)) CD49b and CD34[22–25]. HSC

purities of >50% have been achieved using different sorting strategies[26–30], with a recent report demonstrating that single cell transplantation of HSCs defined by the surface marker combination $CD45^{+}CD150^{+}CD48^{-}EPCR^{high}Sca\text{-}1^{high}$ (ESLAM Sca^{high}) resulted in a purity of 67% in primary recipients and 50% in secondary transplantations[20]. This ESLAM Sca^{high} isolation strategy is the one used in the majority of this thesis for experimental work.

Despite the high similarity between mouse and human HSCs in terms of biological role and cellular function, there are a number of differences which make it challenging to translate all of the research findings from mice into the human system, and mouse HSCs are, as a result, more extensively characterised than their human counterparts.

One important difference concerns the surface marker combinations used for isolating them[31]. CD34 (absent on mouse HSCs and expressed in >99% of human HSCs[32]) was the first such marker discovered to enrich human HSCs and progenitors[33]. In combination with CD34, CD90 (or Thy-1) and CD49f[34] were also introduced as HSC markers while CD45RA and CD38 were identified as markers of more differentiated progenitors which negatively enrich for HSCs[35–37].

Despite these major advances, the best current human HSC sorting strategy allow the isolation of HSCs at a frequency of 1 in 10 from umbilical cord blood (CB) and an even lower frequency from adult BM and PB sources[34]. This, in combination with the high variability across human individuals' genetic, lifestyle and clinical factors, mean that murine models remain a highly valuable research tool for furthering our understanding of HSC biology.

1.1.3 HSC heterogeneity

The classical model of haematopoiesis describes blood production as a tree-like hierarchical structure, where HSCs, residing at the top of the tree, progressively restrict their self-renewal capacity to generate multipotent progenitors and eventually more differentiated mature cells. In early versions of this model, HSCs were considered a relatively homogenous population; however, several studies have revealed significant molecular and functional heterogeneity within the phenotypic

HSC pool, identifying subtypes with differences in cell cycle status, self-renewal abilities and multilineage differentiation output. HSCs are in different states throughout their lifetime: they can remain in the quiescence, or G_0 phase, go into apoptosis, divide symmetrically giving rise to 2 HSCs or to two differentiated cells, or divide asymmetrically giving rise to a one HSC and one non-HSC (Figure 3).

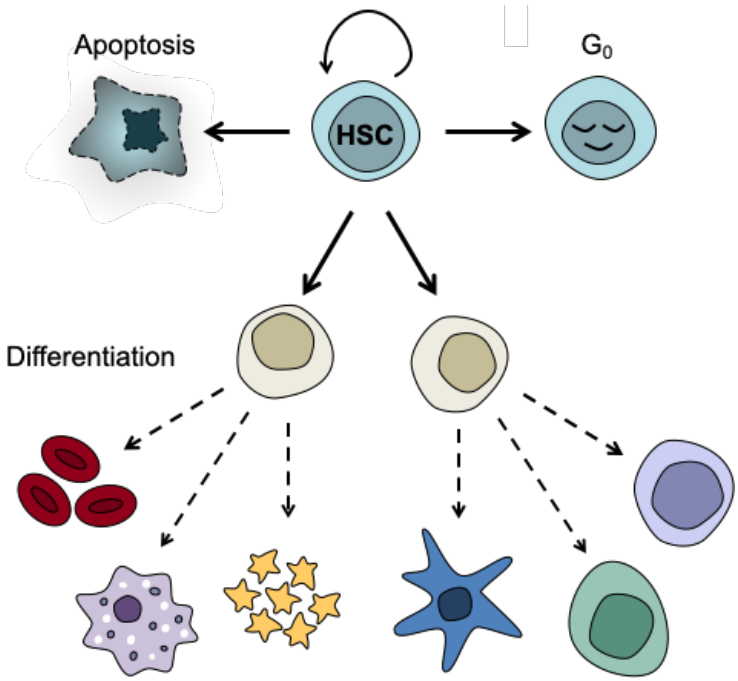


Figure 3. Schematic of HSC fate choices
HSCs can self-renew through symmetric or asymmetric cell division. Dividing symmetrically, it will generate two daughter HSCs, leading to the expansion of the HSCs pool, or two differentiated cells. Asymmetrically, it will give rise to a differentiated daughter cell and to another HSC. Alternatively, the HSC can remain quiescent or undergo apoptosis.

1.1.3.1 Functional heterogeneity of HSCs

The heterogeneous behaviour of HSCs results from the complex interplay of intrinsic and extrinsic variables. The first evidence of heterogeneity in HSCs was shown in the 1960s by Till et al., who reported substantial variability in the number and type of "colony-forming units spleen" (defined as cells capable of giving rise to macroscopic colonies in the spleen of irradiated mice in a 1-2 week period), as well as in the time of detection and the number of secondary colonies they were able to generate[38,39].

These findings were reinforced by analyses of multi-lineage colonies, obtained *in vitro* under the same external stimuli[40–42], and further confirmed by experiments using retroviral marking *in vivo* to track clones over long period of time, thereby demonstrating HSC variability in self-renewal while also reaffirming differences in mature cell production[43,44].

The development of clonal and single cell approaches in purifying stem and progenitor cell populations represented a significant breakthrough in the field and contributed to formally documenting heterogeneity in HSCs. Using limiting dilution assays (LDAs), Muller-Sieburg et al. showed that daughter HSCs deriving from the same clone behave similarly to each other with respect to both self-renewal and lineage contribution[45]. These findings were consistent with the idea that HSC fate is predetermined due to cell-intrinsic mechanisms. Based on the ratio of lymphoid to myeloid mature cells within the total output of donor-derived cells, the HSC clones were classified as myeloid biased (My-bi), lymphoid biased (Ly-bi) or balanced (Bala)[45]. A later study using single cell transplantation outcomes in >100 mice, used a related classification method, where HSC subtypes were classified as α, β, γ or δ by measuring the relative contribution of individual HSC outputs to the total circulating cell numbers (i.e. within the total output of both donor- and recipient-derived HSCs)[46]. α-HSCs give rise to primarily myeloid cells, β-HSCs have a balanced lymphoid and myeloid lineage output, γ generate mainly lymphoid biased but can produce myeloid cells, whereas δ only produce lymphoid cells 16-20 weeks post-transplantation. Both α and β show robust self-renewal and a stable repopulation pattern in primary and secondary recipients, while γ/δ generally fail to repopulate secondary recipients. This is likely reflective of the short lifespan of many myeloid cells and the longer lifespan of lymphoid cells where γ/δ grafts may no longer be producing large numbers of cells at later stages of primary transplantation, and therefore contain few or no HSCs.

Despite the fact that the pattern of mature cell production of the initial single cell transplantation is mostly preserved in daughter HSCs in secondary mice, it is important to note that bulk secondary BM transplantations can contain more than one HSC and some HSC subtypes might be masked in secondary transplantations. Indeed, secondary transplantations of single HSCs isolated from single cell

transplanted primary mice showed that some secondary β clones can be generated by primary α clones and vice versa, demonstrating that the original HSC program is not always maintained[47]. It is also of interest to note that the ratio of α and β-HSCs changes throughout mouse development, with β-HSCs being predominant in the foetal liver (FL, mouse embryonic day (E.)14.5) and α-HSCs only account for 5-10% of all HSCs. Both populations increase at similar rates in the nascent foetal BM (E.18.5) and the α-HSCs become more frequent in the postnatal and young BM and represent the dominant cell type in the older adult BM (>1 year)[47]. It remains unclear if this is the behaviour of two largely independent lineages of HSC subtype or whether β-HSCs eventually turn into α-HSCs.

1.1.3.2 Molecular heterogeneity of HSCs

Despite the advances in methods to isolate HSCs prospectively, such strategies are not able to separate different HSC subtypes, making the study of their molecular differences particularly challenging. Early studies using a high-resolution microfluidic-based single cell qPCR reported the existence of high variability in the gene expression signature of mouse long-term HSCs (LT-HSCs), suggesting that multiple subsets with different molecular fingerprints might exist within the compartment[48]. Similarly, Moignard et al. observed considerable heterogeneity when assessing the expression of key haematopoietic transcription factors in over 500 single haematopoietic stem and progenitor cells (HSPCs)[49].

The advent of single-cell RNA sequencing (scRNA-seq) represented a crucial breakthrough and revealed a number of findings which were previously unattainable by profiling bulk populations or single cells with a targeted gene approach. For HSCs, the first study using scRNA-seq was published by Wilson et al. where single-cell functional assays were combined with single-cell gene expression profiles, resulting in the identification of the core molecular features of HSCs. A molecular signature (MolO gene signature) was defined using the transcriptional overlap between 5 differently defined phenotypic HSC populations and their expected functional content of HSCs. Shortly after this study, Paul et al. identified novel transcription factors involved in myeloid differentiation by profiling HSCs as well as their downstream

progenitors[50] and this was followed by Nestorowa et al. who profiled over 1500 HSPCs, and catalogued the transcriptional changes associated with early differentiation, in particular in the expression of genes associated with metabolism and cell cycle[51].

Together, these studies not only helped identifying new genes and pathways involved in stem cell function and lineage differentiation[50,52], but they also unveiled the high degree of heterogeneity existing within various stem and progenitor cell fractions and confirmed that stem cell differentiation is a gradual molecular process (i.e., a continuum) and not a stepwise progression through differentiated progenitors with fixed transcriptional profiles[53]. The source or relevance of this molecular heterogeneity has yet to be defined, nor whether different factors contribute to it during development or via their residence in different tissues or haematopoietic niches.

1.1.4 The haematopoietic stem cell niche

While many HSC decisions have been demonstrated to be intrinsically governed, there is growing evidence that stem cell decisions also depend on signals coming from the surrounding microenvironment, also known as the "blood stem cell niche". Ray Schofield first proposed this concept in 1978, when he described it as the anatomic environment in which maturation of stem cells is prevented and the "stem-ness" properties are preserved[54]. Although the concept of a "niche" was initially proposed in vertebrates[39,55,56], studies in *Drosophila* and *Caenorhabditis elegans* facilitated the understanding of stem cell behaviour, where stem cells reside in specific constricted locations (e.g. the ovary in *Drosophila* and the gonads in *C.elegans*)[57,58]. Taking advantage of the simplicity of these models, it was formally shown that absence of specific extrinsic regulators in niche cells lead to stem cell pool abolishment. Similarly, the overexpression of such factors could drive the proliferation of immature precursors and the creation of ectopic niches. This evidence enforced the idea that the cell-extrinsic cues from the surrounding environment can play a major role in regulating stem cell fate despite having the same intrinsic components in the stem cells themselves.

In the mammalian haematopoietic system, a higher level of complexity exists due the blood being a dynamic tissue. HSCs do not necessarily remain in a fixed niche, but instead they are capable of circulating, making their interactions with the surrounding environment dynamic rather than static. This is further complicated by the fact that (as described in section 1.1.3) the HSCs are functionally and molecularly heterogeneous, suggesting the possibility that multiple specialised niches exist for distinct subpopulation of HSCs[59–61]. Advancements in imaging tools and the discovery of new markers of HSCs and niche cells have allowed a better understanding of the HSC microenvironment and the field has grown rapidly in recent years. In the next section, I review the main findings of the past several years in the understanding of the BM niche and in particular how its cellular components and secreted factors contribute to regulating HSC function and behaviour.

1.1.4.1 The endosteal and the vascular niche

In 1972, Knospe and colleagues provided the first evidence that BM sinusoidal vascular regeneration occurred in areas of damaged BM[62]. However, the existence of a vascular niche for HSCs was only recognised much later when immuno-histochemical analyses showed that about 60% of Sca-1^{+}Lin^{-}CD150^{+}CD48^{-} (SLAM) HSCs localised in the proximity of sinusoidal endothelial cells (SECs)[27]. Moreover, HSCs seem to preferentially engraft in the BM vascular domains, when transplanted in recipient mice[63,64]. Nestin positive perivascular cells[65] and Leptin receptor positive (LepR^{+}) perivascular cells[66] express high levels of key HSC niche factors, such as Stem cell factor (SCF) and CXCL12, as well as other regulatory factors such as osteopontin (OPN), Interleukin-7 (IL-7), angiopoietin-1 (ANGPT1)[67] and deletion of *Scf* and *Cxcl12* from LepR^{+} cells results in HSC depletion and perturbed haematopoiesis. Moreover, angiopoietin-mediated signalling was reported to promote the interaction of *Tie2*-expressing HSCs with surrounding mesenchymal cells and the extracellular matrix (ECM), and such interactions directly contributed to protecting HSCs from exhaustion[68].

The first indication that HSCs might be physically located close to the bone surface (endosteum) and that an endosteal niche might exist was reported in 1975. Brian Lord and colleagues, by flushing the centre of a mouse femur and comparing it to the

edges, showed that a considerably higher concentration of colony-forming-unit (CFU) cells resided closer to the bone surface compared to the centre of the BM cavity[69,70]. Shortly thereafter, Dexter et al. demonstrated that BM-derived stromal cells were able to maintain granulopoiesis *in vitro*[71]. This was followed by data from numerous groups, showing that multiple population of cells isolated from the BM could support lymphopoiesis and myelopoiesis when in culture with HSCs[72–76]. In 1996, Russel Taichman hypothesised that BM osteoblasts were critical to support HSC *in vivo*, and showed that human osteoblasts were able to promote human $CD34^+$ expansion in culture[77]. Further studies in mice formally demonstrated that most primitive haematopoietic cells resided close to endosteum, suggesting that the endosteal and osteoblastic cells constitute a specific HSC microenvironment *in vivo*[78,79]. This observation was also confirmed in more recent studies using three-dimensional imaging of HSCs in whole mouse bones and supported by evidence showing that genetic manipulations of osteoblastic cells can alter HSC function[80,81].

In the past few years, molecular profiles of niche cells were employed by a number of groups, in order to further investigate which extrinsic molecular factors are involved in regulating HSC function *in vivo*[82,83]. This approach helped to characterise a number of candidate BM niche-expressed factors that may be required to maintain and expand HSCs and preserve them from DNA damage *ex vivo*, as well as identifying major sources of niche-derived factors involved in regulating HSC behaviour. Altogether, this evidence demonstrates that the cellular environment is emerging as a fundamental regulator of haematopoiesis, with impacts on the size and lineage distribution of the blood system, the cellular fate of HSCs and/or immature progenitors, and the remodelling of the BM niche that support these HSC outcomes[84].

1.1.4.2 Inflammatory factors secreted in the HSC niche

Many of the niche cells located in the BM secrete cytokines and growth factors which can modulate HSC self-renewal and differentiation *in vivo*. Recent evidence suggests that HSCs might be direct targets of inflammatory signalling and that such signalling may also contribute to HSC regulation during homeostatic haematopoiesis, by promoting survival and expansion of committed progenitors or contributing to maintenance of their quiescence state. In addition, there is strong evidence that

certain cytokines indirectly affect HSCs through alteration of the BM microenvironment during inflammation. This section will review some of the main environmental molecules and cytokines that have been proved to directly or indirectly act on HSCs, their effect on HSC function and differentiation and their potential contribution in regulating HSC fate.

1.1.4.2.1 Interferons

Interferons (IFNs) play a critical role in the activation of the innate and acquired immune system, as well as in the response to viral and bacterial infections, and tumour cells. Multiple lines of evidence have shown that IFNs can act as moderators of haematopoiesis.

Essers et al. reported that *in vivo* stimulation of IFN-α, a type I IFN, leads to increased HSC proliferation *in vivo*, via the activation of Signal transducers and activators of transcription 1 (STAT1) and Akt (or protein kinase B (PKB)) pathways, resulting in impaired HSC function when the exposure is prolonged[85]. Similarly, loss of interferon regulatory factor-2 (IRF2), a transcriptional repressor of IFN-α signalling, results in the increase of HSC proliferation and reduction in self-renewal activity[86]. IFN-γ has been shown to induce HSCs to exit from dormancy and proliferate. As reported by Goodell et al, the activation of IFN-γ in response to chronic bacterial infection leads to increased HSC proliferation *in vivo*[87]. In humans, IFN-γ showed an inhibitory effect on $CD34^{+}CD38^{-}$ CB cells, with reduction of their ability to support multi-lineage haematopoiesis, when transplanted into NOD scid gamma (NSG) mice[88]. Similarly, loss of IFN-γ in a knock-out (KO) mouse model results in increased HSC repopulating capacity compared to wild type (WT) HSCs[89]

Altogether, these findings suggest that IFN-dependent inflammatory signals may play a central role in regulating HSC behaviour and in inducing HSCs to exit their quiescent state. However, the exact mechanisms through which IFNs affects HSC function has yet to be determined.

1.1.4.2.2 Thrombopoietin

Thrombopoietin (TPO) is the primary regulator of platelet production from BM megakaryocytes and is responsible for maintaining platelet homeostasis[76,90]. Via its receptor, MPL, TPO regulates a variety of downstream signalling pathways, promoting cell survival and proliferation. The TPO receptor is primarily expressed on megakaryocytes and platelets and therefore depletion of MPL only results in reduction of these populations while the other mature cells remaining unaffected[91]. However, TPO and MPL KO mice show a significant decrease in the number of repopulating HSCs suggesting that TPO signalling could be essential in maintaining HSC number and function[91]. Similar observations can be drawn in humans, where TPO signalling has also been linked to HSC regulation. In fact, patients with loss-of-function mutations in *Mpl*, develop congenital amegakaryocytic thrombocytopenia (CAMPT), and progressively BM failure[92]. *In vitro* studies show that TPO stimulation of HSCs result in increased survival and repopulation activity, especially when in combination with SCF and IL-3[93,94]. In support to this notion, Wilkinson et al. recently reported that high TPO (100ng/mL) in combination with low SCF (10ng/mL), without the addition of any other soluble factors, can achieve 200-900-fold increase in functional HSCs over a period of a month[95].

Overall, these results demonstrate that although TPO is primarily involved in megakaryopoiesis, a crucial role in regulating HSC function and maintenance has been shown in both mouse and human system.

1.1.4.2.3 Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) is a potent HSC mobilising agent, produced by a number of haematopoietic and BM stromal cells in response to inflammatory signals (e.g., viral and bacterial infections). Although it is mainly involved in regulating neutrophil production, there is considerable evidence that G-CSF is also able to indirectly induce mobilisation of HSPCs[65,96], by inducing changes in stromal cells implicated in HSC maintenance (such as Nestin positive stromal cells). Administration of G-CSF *in vivo* results in an increase in phenotypic HSCs in the BM but these HSCs have significantly reduced function compared to untreated mice[97–99]. In addition, G-CSF receptor signalling appears to have an important effect on HSCs

where G-CSF receptor KO mice show normal numbers of phenotypic HSCs but defects in their repopulation activity[100]. Conversely, ectopic expression of G-CSF receptor confers a clonal advantage to HSCs, following G-CSF stimulation[101]. Overall, this evidence suggests that G-CSF plays an important role in maintaining HSC function under homeostatic conditions, with ectopic G-CSF expression resulting in an alteration in the BM microenvironment and subsequent impaired HSC function.

1.1.4.2.4 Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF-α) is one of the most intensively studied pro-inflammatory cytokines. It is mainly produced by monocytes and macrophages and it is involved in a variety of inflammatory processes, including cell cycle regulation and cell differentiation[102].

However, its role in HSC regulation remains controversial in both the mouse and human systems. Conflicting results have been reported for the effect of TNF-α on human HSC growth and proliferation *in vitro*[103,104]. Similarly, studies using the TNF-α receptor KO mouse show opposite effects as to whether TNF-α influences mouse HSC survival positively[105] or negatively[106]. In addition, depending on the experimental setting, both HSC engraftment promoting and inhibitory effects are observed[106,107].

A recent study from Passagué et al. reported a differential effect of TNF-α on HSCs and mature progenitors, showing that TNF-α supports survival of HSCs, while inducing apoptosis in more committed progenitors and mature cells. Administration of TNF-α *in vivo* induces a transient increase in HSC numbers and subsequent induction of HSCs towards myeloid cell production[108].

Overall, the exact role of TNF-α on HSC survival requires further clarification and would be furthered bolstered by studies identifying the pathways through which TNF-α might regulate HSC function and differentiation.

1.1.4.2.5 Toll-like receptors

Toll-like receptors (TLRs) belong to a family of transmembrane pattern recognition receptors (PRRs), which can detect pathogen-associated molecular patterns (PAMPs; e.g. Lipopolysaccharides (LPS), peptidoglycans), and are involved in both innate and adaptive immune response to pathogens[109]. Both human and mouse HSCs have been

shown to express TLRs[110–112]. Mouse HSCs express TLR2 and TLR4 *in vitro* and stimulation with LPS leads to enhanced cell cycling and myeloid differentiation[110]. Moreover, chronic exposure to LPS *in vivo* results in increased HSC cycling and reduced self-renewal[113]. In human, prolonged activation of TLRs in $CD34^+$ induce proliferation and myeloid differentiation. The idea that microbial components could be cues for HSC function and that such signals may help regulate haematopoietic cell production has been described in the context of systemic bacterial infection inducing "emergency granulopoiesis", a characteristic haematopoietic response programme characterised by systemic signs such as neutrophilia, blood leucocytosis, and the appearance of immature neutrophil precursor cells in the PB[114].

The activation of emergency granulopoiesis requires pathogen sensing through the activation of PRRs, which leads to a cascade of events, including the production of cytokines which can stimulate granulopoiesis (e.g., G-CSF, IL-3, IL-6, and fms-like tyrosine kinase-3 ligand (FLT3-L)). In addition, because HSCs also express PRRs, HSCs can directly sense systemically spreading pathogens, and PRR ligation can stimulate their proliferation and granulocytic differentiation[115]. Altogether, these observations demonstrate that HSCs can respond to inflammatory stimuli in both steady-state and emergency granulopoiesis and enforce the idea that extrinsic inflammatory factors can both directly and indirectly regulate HSC function.

1.1.4.2.6 Stem cell factor

Stem cell factor (SCF), also called Steel factor (SF)[116], Mast Cell Growth Factor[117] (MGF), and Kit-Ligand[118] (KL) is a cytokine produced by BM endothelial and mesenchymal stromal cells, including adipocytes and LepR-expressing perivascular cells[119]. Both SCF and its receptor c-Kit play a critical role in the regulation of haematopoiesis. Mutant Steel (*Sl*) mice are characterised by anaemia, reduced gonad size and reduced hair pigmentation. The majority of the homozygous mice die perinatally due to macrocytic anaemia[120]. Similarly, homozygous white-spotting (*W*) mice, with loss of function mutations at the c-Kit-receptor locus, are severely anaemic, sterile and have reduced number of mast cells[121,122].

The idea that SCF may act directly on HSCs was already proposed in the early 1960s by McCulloch and Till. By performing transplantation of haematopoietic cells derived from mutant *Sl* mice into WT recipients and vice versa, they observed that no

differences in colony forming efficiency was observed in WT recipients. However, fewer colonies were detected when WT cells were transplanted into *Sl* recipients suggesting that the defect exerted by the Sl mutation affects the haematopoietic environment and it is not intrinsic to the colony-forming cells[123]. Interestingly, transplantation of WT foetal liver cells, as well as BM cells from *Sl* mutant mice, into the *W* recipients could partially or entirely normalise the anaemic phenotype[123].

SCF has been shown to stimulate proliferation of both human and mouse HSCs[124,125]. Supporting this notion, *in vivo* administration of SCF in mice drives both peripheral blood and marrow cellularity increases within the first 7 days post-injection[126].
In vitro studies have shown that SCF, alone or in synergy with other cytokines (FLT-3, IL-3, IL-6, TPO) acts on HSCs and progenitor cells, by promoting their expansion in culture for up to 14 days and maintaining engraftment capability[127–130]. Similar results have been obtained with SCF in combination with IL-11[131–134]. On its own, SCF increases HSC survival compared to media without any cytokines.
More recent studies on single HSCs ($Lin^{neg}CD45^{mid}Rho^{neg}SP$) have shown that a high concentration of SCF promotes self-renewal, whereas HSCs cultured in 30-fold lower amount of SCF show a significative reduction in reconstitution capacity[26].

A series of SCF concentration were tested by Wilkinson et al., showing that stimulation with high concentration of SCF (100ng/mL) achieve higher chimerism in competitive transplantation, when compared to low concentration of SCF (1ng/mL). However, when in combination with high concentration of TPO (100ng/mL), lower concentration of SCF (10ng/mL) led to increased HSC expansion[95].

Interestingly, another study showed that abrogation of SCF/c-Kit signalling, via inhibition of lipid raft clustering (including the c-Kit receptor), results in HSCs ($Lin^{-}Sca1^{+}c\text{-}kit^{+}$(LSK) $CD34^{neg}$) remaining as single cells for up to 10 days *in vitro* while maintaining their ability to give rise to multi-lineage reconstitution in primary transplantation[135]. Altogether, these results suggest that SCF is required for HSC proliferation and self-renewal but may be dispensable for the maintenance of their "stemness".

1.1.4.2.7 Transforming growth factor beta

A number of studies have described transforming growth factor beta (TGF-β) as a key regulator of haematopoiesis. *In vitro* studies have shown a potent inhibitory effect of this cytokine on both human and mouse HSCs[136–138], whereas more committed progenitors are less affected or in some cases induced to proliferate[137,139]. In addition, treatment of human HSCs with anti-TGF-β antibodies seems to induce quiescence exit and increase the frequency of colony formation[140,141]. Similarly, murine BM cells treated with anti-TGF-β prior to transplantation show enhanced repopulation ability[142]. A recent study from Goodell et al. showed that distinct haematopoietic stem cell subtypes respond differently to TGF-β1, suggesting a possible differential regulation of HSC subtype activation[28]. Together, these results suggest an important role for TGF-β in regulating HSC quiescence and proliferation and highlight the possibility of using its signalling for *ex vivo* activation and expansion protocols.

1.1.4.2.8 Gfp130 family members

Previous reports have shown that IL-6 or IL-11 stimulation enhances HSC expansion via activation of the glycoprotein 130 (gp130) receptor, both *in vitro* and *in vivo*[132,143]. Often used in conjunction with IL-3, SCF, TPO and G-CSF, IL-6 and IL-11 have synergistic mitogenic effects on HSC proliferation[144,145]. Interestingly, IL-3 has been reported to have a negative effect on proliferation when used *in vitro* without IL-6[129]. More importantly, LSK cells fail in their reconstitution capacity when transplanted after 14-day culture with SCF and FLT-3L alone[134], suggesting that inclusion of IL-6 or IL-11 is essential to retain HSC stemness.

Overall, it is clear from loss-of-function studies that cytokines play an important role in maintaining lineage output during homeostatic haematopoiesis and under stressed conditions, by promoting survival and expansion of committed progenitors[146]. However, while the function of these factors on haematopoiesis has advanced, a number of questions still remain. The cellular targets of individual cytokines are not always clear (as evidenced for TPO/MPL above with dual roles in HSCs and megakaryopoiesis) as well as their implications for the haematopoietic system functionality. Further combinatorial studies on how individual cytokines affect HSC function *in vivo* and *in vitro* might help to better clarify their role in

determining HSC fate in homeostasis, as well as their contribution in the context of dysregulated haematopoiesis.

1.1.5 Significance of *in vitro* expansion of HSCs

The ability to self-renew while maintaining multipotency makes HSCs an essential component for the treatment of patients with a number of haematologic disorders. Since their haematopoietic system has been compromised by the disease, as well as by the chemo- radio- or immune-therapy used to eliminate the malignant cells, a BM transplantation is often the only curative therapy with the ability to rebuild the blood system. However, the number of donor cells required for a successful allogeneic transplant is very large[147]. This, in combination with the lack of human leukocyte antigen (HLA)-matched donors and the low number of stem cells available from common HSC sources (BM, mobilised PB, umbilical CB) represent a serious limitation to HSC transplantations[148,149]. In addition, patients who receive allogeneic transplants may exhibit a rejection of the transplanted cells, or experience graft-versus-host disease (GVHD), disease relapse, and/or side effects of the conditioning regimen used[147].

Since HSCs are able to expand substantially in their native niche, trying to mimic the environment has been a major goal for the development of protocols to successfully expand HSC populations prior transplantation[149], and extensive work has been undertaken in order to guarantee higher, on-demand, availability of HSCs for the treatment of haematological diseases. In the next section, I will describe current strategies (and challenges) to expand HSCs *in vitro*.

1.1.5.1 Gene modification efforts

Valuable knowledge about the factors affecting self-renewal has been gained from studies on gene manipulation. These have identified a number of proteins that play an important role in regulating HSC self-renewal, including transcription factors, cell cycle regulators and epigenetic modifiers. One of the first described genetic studies for expanding HSCs was *Hoxb4* overexpression, where ectopic expression resulted in 40-fold expansion of transplantable HSCs and did not lead to the development of leukaemia[150,151]. Delivering the fusion protein TAT-HoxB4 also contributed to HSC

expansion but with a much lower expansion levels compared with ectopic protein expression[152].

Other members of Hox family (e.g. HoxA4, HoxB9, HoxB6) have also been shown to affect HSC fate[153–156]. Perhaps most impressive is the over-expression of a NUP98-HOXA10 homeodomain fusion protein which was able to sustain long-term engraftment in transplanted recipients, with a balanced myeloid and lymphoid cell production, reaching more than 1000-fold expansion of HSCs in short-term *in vitro* cultures[157–159]. Similarly, Denault et al. reported a number of Hox target genes, including *Fos, Tcfec, Hmgb1, and Sfpi1*, whose over-expression by retroviral transduction *in vitro* could enhance HSC activity in transplantation experiments[160].

In addition to these studies, several miRNAs have been reported to modulate HSC self-renewal, including miR-29a and the miR-125 family[161–165]. Finally, overexpression of epigenetic regulators, such as members of the Polycomb-group, have been shown to modulate HSC activity by increasing self-renewal or preventing stem cell exhaustion and genetic activation of Notch and Wnt[166] signalling has been shown to promote HSC self-renewal *ex vivo*.

Despite the *ex vivo* expansion reported after ectopic expression of such cell-intrinsic genes, introducing genetic materials is not ideal in clinical protocols since the potential of cell modifications and malignant transformations or stem cell exhaustion cannot be excluded[149].

1.1.5.2 Co-cultured cells

In the early 90's, a number of studies achieved the clonal expansion of murine HSCs using stromal-based long-term culture without the addition of exogenous growth factors[71,167–170]. More recently, preclinical studies revealed that direct interactions between mesenchymal stromal cells (MSCs) and CB $CD34^+$ cells *ex vivo* result in expansion of the total number of cells[171–174]. Activation of Notch pathway in BM endothelial cells has been shown to increase the number of CB $CD34^+$ in culture, and the transplant of these expanded cells seems to shorten the time of neutrophil recovery in a phase I clinical trial[175–177].

However, such recovery is transient, suggesting either an immune-mediated rejection in the transplanted patients or a potential loss of stem cell self-renewal during culture,

which then results in the expansion of short-term repopulating HSPCs instead of LT-HSCs[171,175,178,179]. Together, this current evidence supports the notion that these culture conditions might be suboptimal for stimulating HSC self-renewal or long-term survival and, importantly, incorporation of additional cell types would unavoidably increase the complexity of transplantation procedures.

Stroma-noncontact culture where cells are cultured separately using a trans-well plate have been developed by using various cell lines (e.g. the embryonic stromal cells UG26[180] and EL08-1D2[181,182]) which are able to maintain adult BM HSCs. These cultures support the notion that HSC maintenance *in vitro* can be achieved by the use of soluble factors alone, although early molecular profiling efforts have yet to identify the exact combination of factors that can replicate the potency of the co-culture or conditioned medium experiments.

1.1.5.3 Cytokines, recombinant grow factors and small molecules

Later and more recent attempts to expand HSC outside the body focus on stromal-free cultures and the use of haematopoietic cytokines, which have been shown to support HSCs *in vivo* in a number of different studies[183]. Different combinations of recombinant growth factors and cytokines, small molecule agonists and antagonists, have been assessed in order to develop clinically applicable regimens for the maintenance or expansion of stem cells, by preserving and improving their engraftment potential.

1.1.5.3.1 Growth factors and cytokines

A number of growth factor combinations have been reported to sustain HSC maintenance in both mouse and human, including fibroblast growth factor (FGF1)[184], insulin-like growth factor 2 (IGF2)[185], insulin growth factor-binding protein 2 (IGFBP2)[186], Wnt3a[166], pleiotrophin[187], members of the Angpt-like family, and immobilized Delta1 (DL1)[188].

In the mouse, SCF and IL-11 or SCF and TPO have been shown to support HSC survival and proliferation and contribute to repopulating activity maintenance[135,189–191].

These molecules are also used in standard human HSC culture systems, although usually in combination with FLT3-L, IL-6[192–194]. TPO has also been reported to support long-term culture, although, when added to the medium in combination with SCF, FLT3-L, IL-3, IL-6, G-CSF and nerve growth factor (NGF), it did not give additional benefit[192]. Importantly, cytokines are usually added in combination with foetal bovine serum (FBS) or human serum, or bovine serum albumin (BSA) and insulin[195,196].

Such culture conditions achieve HSC maintenance *ex vivo* for 1-2 weeks but provide minimal HSC expansion, suggesting that the addition of cytokines has only limited capacity to maintain long-term self-renewal.

A major breakthrough was achieved in a recent study from Wilkinson and colleagues[95], who described a new approach to culture HSCs, based on the novel use of polyvinyl alcohol (PVA) as a substitute for serum albumin, and on the frequent complete replacement of the culture media. This strategy is based on the idea that the old media is being "conditioned" and contains soluble factors secreted by the cells that could further promote cell growth and differentiation and therefore HSC loss. Interestingly, this condition does not require the addition of any extra soluble factors apart from SCF and TPO, and it achieves 200-900-fold increases in functional HSCs over a period of a month[95].

1.1.5.3.2 Small molecules

To date, a number of small molecule agonists of HSC self-renewal have achieved significant expansions of human HSCs. In one of the earliest and most significant advances in human HSC expansion in 2010, Boitano et al. identified a purine derivative, StemRegenin 1 (SR1), as a promoter of *ex vivo* expansion of PB and CB $CD34^+$ cells after screening over 100,000 small molecules. Culture of HSCs with SR1 increased the number of $CD34^+$ 2.6-fold and 73-fold, after 7 and 21 days in culture respectively, compared with control cells. SR1 expanded HSCs retained multi-lineage potential, with 65-fold increase in CFUs, 17-fold increase in cell numbers that were capable of late engraftment in NSG mice[197] and, most importantly, in subsequent studies, sustained engraftment in a substantial proportion of patients[198]. However, the majority of the expansion was demonstrated to be in the committed progenitor

fraction ($CD34^+CD133^+CD90^-$ and $CD34^+CD133^-CD90^-$) as opposed to the LT-HSC fraction.

In another landmark study in 2014, Fares et al. identified UM171, a compound which targets more primitive cells than those targeted by SR1, significantly increasing the frequencies of human $CD34^+CD38^-CD90^+CD45RA^-CD49f^+$ compared to SR1 using a fed-batch culture system. Importantly, the *in vivo* data showed that CB cells cultured in presence of UM171 retain full functional capacity to repopulate both primary and secondary irradiated recipients[148].
In addition to these molecules there have also been advances using MPL agonists and small-molecule mimics of TPO, which have been demonstrated to promote human HSC self-renewal *ex vivo*[199,200]. These small molecules target human proteins and pathways and therefore they have been almost exclusively studied in human culture to date.

Despite these advances, the development of robust culture strategies to maintain functional human HSCs in culture has not been achieved. This is at least in part due to the difficulties in deciphering the molecular mechanisms controlling HSC expansion *ex vivo* since the vast majority of cells created in such cultures are non-HSCs, thereby precluding the molecular profiling of highly purified HSCs in the absence of a haematopoietic niche. The identification of key molecules and networks important for self-renewal *in vitro* could significantly improve our ability to expand HSCs and eventually lead to the development of compounds that would specifically target crucial self-renewal factors for the eventual scale-up of HSC production as a base product for a wide array of cell and gene therapy protocols.

1.2 The role of extrinsic microenvironment in driving aberrant (or malignant) haematopoiesis

1.2.1 Extrinsic regulators of haematological disease

A greater understanding of the extrinsic inflammatory mediators and key supportive factors required for physiologic haematopoiesis and HSC function has not only helped improving culture conditions for their *ex vivo* expansion, but it also created a new paradigm for thinking about haematological disease. A sustained inflammatory microenvironment provides a constant supply of various cytokines, chemokines and growth factors, which are able to modulate anti-tumoral responses and protect damaged or infected tissues, by recruiting immune cells to help restore their normal function.

As these molecules and their associated receptors provide signals for important processes, it is not surprising that alterations in cytokines, their receptors, and/or the signalling pathways that they mediate, are involved in a wide variety of inflammatory diseases and cancer. Moreover, chronic inflammation can result in the alteration of pro- and anti-inflammatory cytokines balance or their relative concentration, and in the constitutive activation of immune cell responses, leading to genomic instability and to cell transformation and malignancies[201]. Recent studies emerging from the field of pre-leukaemic disease and leukaemia suggest that the inflammatory microenvironment might play a crucial role in the pathogenesis and progression of these diseases[202–205].

High levels of inflammatory cytokines have been observed in a number of haematological malignancies[206–211] and several lines of evidence support the potential association of such cytokine deregulation with pathogenesis, disease progression, and patient survival. TNF-α, IL-6 and IL-10 serum levels are increased in leukaemia patients compared to age-matched healthy controls[212]. Furthermore, increases in pro-inflammatory cytokines has been associated with adverse clinical outcomes in elderly individuals[213,214].

Several reports have shown a dynamic interaction between malignant clones and their niche. The mutant clones possess the ability to remodel the surrounding environment to create a reinforcing niche that supports the malignant cells preferentially to the non-mutant cells, impacting their future survival and growth through the production of cytokine and chemokines[215].

However, the specific role of the immune components in early stages of haematological malignancies is not well understood, due to the malignant cells belonging to the immune system itself and the consequential difficulty in dissecting out particular molecules and whether they are secreted by and/or attached to different cells.

Whereas section 1.1 was focused on the importance of extrinsic regulators and their contribution to HSC function in the context of normal haematopoiesis, the following sections will discuss how alterations of such factors can play a central role in dysregulated haematopoiesis, and in particular the potential role of cytokines and chemokines in a class of clonal preleukaemic disorders called myeloproliferative neoplasms (MPNs).

1.2.2 The BCR-ABL negative myeloproliferative neoplasms (MPNs)

The myeloproliferative neoplasms (MPNs) are a group of clonal disorders originating in the HSPC compartment and characterised by alterations in mature blood cell production[216]. Within the MPNs, chronic myeloid leukaemia (CML) was the first disease to be associated with a genetic alteration: the BCR-ABL fusion gene, which most commonly results from the Philadelphia chromosomal translocation (Ph)[217]. This single genetic aberration is found in all patients with CML and its existence as a direct and unique genetic abnormality represented an incredible candidate for targeted therapy. The development of effective therapeutic inhibitors made CML a paradigm of rational drug design in human malignancies and imatinib was the first signal transduction inhibitor used in clinic[218–221].

The identification of the Janus Kinase 2 (JAK2) V617F mutation in 2005[222–227] and its identification in the large majority of BCR-ABL negative MPN patients provided a

strong rationale for the development of pharmacologic inhibitors that target this pathway. The first class of inhibitors were JAK/STAT inhibitors, typified by ruxolitinib (a JAK1/2 inhibitor), which is administrated to patients with intermediate- to high-risk myelofibrosis with some clinical benefit[228], including a modest increase in survival and reduction in spleen volume. That said, the positive effects of these inhibitors might be partially due to their anti-inflammatory effect rather than a specific targeting of the mutant clone[229].

The three main BCR-ABL negative MPNs include:

- PV, polycythaemia vera, primarily associated with increased numbers of red cells in the peripheral blood and increased erythroid, megakaryocytic and granulocytic progenitors in the BM. Major criteria for PV diagnosis require haemoglobin (Hb) level greater than 16.5 grams per decilitre (g/dL) in women and 16.5 g/dL in men, or haematocrit (HCT) level higher than 49.5 percent (%) and 55.5% in women and in men respectively[230].

- ET, essential thrombocythaemia/thrombocytosis, characterised by an increased number of platelets in the peripheral blood (above 450,000 per microliter of blood) and an increase in megakaryocytes in the BM.

- MF, myelofibrosis, correlated with frequent splenomegaly and progressive anaemia, as a consequence of extra-medullary haematopoiesis and BM fibrosis. It can occur as primary disease (primary MF (pMF)) or as a consequence of post-chronic disease (PV or ET) transformation (secondary MF (sMF)) (Figure 4).

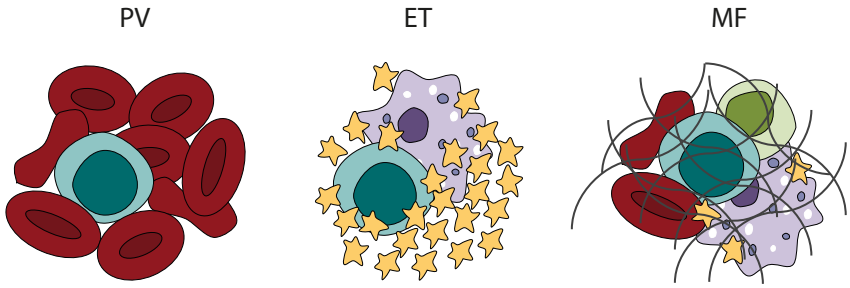


Figure 4. BCR-ABL negative myeloproliferative neoplasms.
The BCR-ABL negative myeloproliferative neoplasms (MPNs) can be classified into three different subgroups: polycythaemia vera (PV), mainly associated with increased numbers of red cells in the peripheral blood, essential thrombocythaemia (ET), characterised by an increased number of platelets and megakaryocytes in the peripheral blood and in the BM, and myelofibrosis (MF), correlated with progressive anaemia, frequent splenomegaly and BM fibrosis. Illustration by Mairi Shepherd.

All three groups are closely related and share a propensity for thrombosis and haemorrhage, but a substantial diversity of symptoms exists between patients. Some patients with PV and ET develop MF, which is considered the most severe subtype and has the lowest survival rates[231]. All MPN patients, especially MF patients, show an increased tendency toward leukaemic transformation, with around 12% of patients eventually developing acute myeloid leukaemia (AML)[232] and, much less commonly, an occasional transformation to acute lymphoblastic leukaemia (ALL)[233].

At present, available treatments for MPNs are generally aimed at reducing the increased number of blood cells in circulation (through cytotoxic agents such as hydroxyurea and hydroxycarbamide) with a minority of (mostly younger) patients being offered a potentially curative therapy via allogeneic haematopoietic stem cell transplant (Allo-HSCT). Pegylated IFN2α is a promising therapy in patients who are not likely to undergo Allo-HSCT. Treatment with pegylated IFN2α show an improvement in the overall survival and leukaemia-free survival, correlating with the duration of the IFN2α therapy, and it's accompanied by more than 50% allele burden reduction in over 50% of the treated patients[234]. In many cases, treatment with aspirin seems to be effective for alleviating microvascular symptoms, such as erythromelalgia and headaches, possibly preventing vascular events in JAK2-mutated ET[235].

MPNs represent a useful model for understanding deregulated haematopoiesis since they arise from a single cell (promptly permitting clonal analysis), and they are chronic diseases, readily facilitating dissection of disease evolution over a number of years. Additionally, no differentiation block is observed in MPNs, which gives access to an early stage of malignancy that is not available in other tissues[236]. Moreover, as MPNs are genetically less complicated that many other malignancies[237], they can be used as a system in which to study the effect of mutation combinations[238].

1.2.3 Genetic mutations in MPNs

In the majority of cases, MPNs are the result of mutations in the HSC compartment, which lead to aberrant progenitor production and risk of leukaemic transformation. In MPNs, the most common driver mutations involved in normal stem cell transformation can be classified into 2 different groups:

- Mutations in signalling proteins such as JAK2, Calreticulin (CALR), and MPL;
- Mutations in epigenetic regulators of DNA methylation (such as Ten-Eleven Translocation (TET2), DNA methyltransferase 3A (DNMT3A), and Isocitrate dehydrogenase 1/2 (IDH1/2)) and of chromatin structure (such as Enhancer of zeste homolog 2 (EZH2) and ASXL1); (Figure 5).

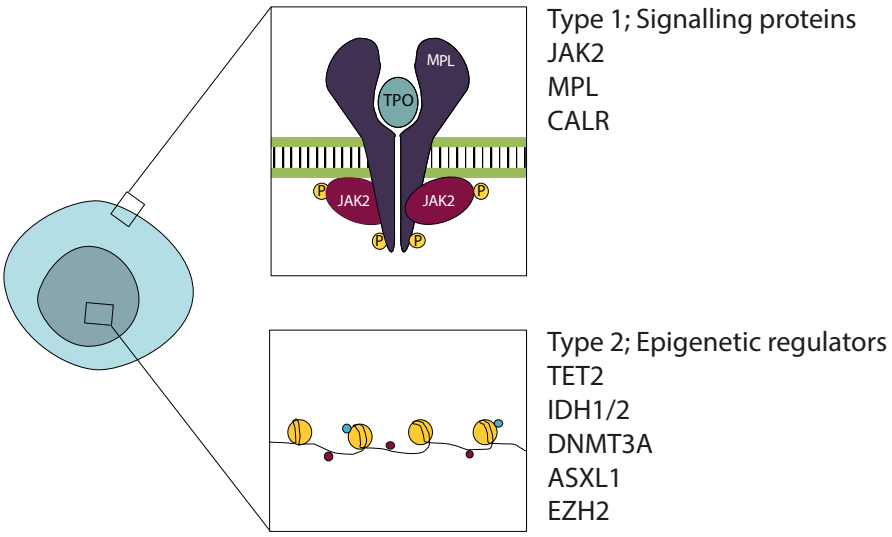


Figure 5. Common somatic mutations in MPNs.
The most common mutations in MPNs can be classified into Type 1 (signalling proteins) and Type 2 (epigenetic regulators). Illustration by Mairi Shepherd.

The JAK2V617F mutation is the most common mutation in MPNs. It leads to the hyper-activation of JAK2 pathway caused by a single amino acid substitution in the pseudo-kinase (JH2) domain, which under normal circumstances is involved in the downregulation of the kinase activity. This results in ligand-independent activation of JAK2 signalling and/or hyper-sensitivity of haematopoietic cells to growth factors and cytokines[239]. Somatic gain-of-function mutations have also been identified in exon 12 of JAK2 (V617F is in exon 10) in the rare JAK2V617F-negative PV. Exon 12 mutations are not correlated with ET and MF, with the majority of patients exhibiting a specific clinical phenotype, with variable leucocytosis and polycythaemia but not thrombocytosis[225]. It is possible, however, that JAK2 exon 12 PV may develop secondary myelofibrosis[240]. The JAK2V617F mutation has also been reported, although at lower frequencies, in other myeloid malignancies, including chronic myelomonocytic leukaemia (6%), BCR-ABL-negative CML (19%) and myelodysplastic syndrome (MDS) (3%), while it is almost absent in the lymphoid malignancies. Mutations in JAK2 are also commonly associated with age-related clonal haematopoiesis (ARCH), defined as the expansion of HSPC clones in individuals without a diagnosis of hematologic malignancies[241]. Although the majority of individuals with ARCH do not develop any clinical features, an increased risk of developing MDS and other blood cancers, is present.

Subsequent to the JAK2 mutations, many other mutations have been identified. Mutations that also target the JAK-STAT cytokine signalling pathways have been found in the TPO receptor, MPL [242], and the endoplasmic reticulum (ER) chaperone CALR[243,244]. JAK2, CALR, or MPL mutations are nearly always mutually exclusive and they represent the most common mutations in MPNs[245]. CALR-mutant ET and MF patients show a milder phenotype compared with JAK2V617F and MPL, with a significantly longer overall survival. Loss of function mutations in the adaptor protein Sh2b3 (LNK), somatic mutations in suppressors of cytokine signalling (SOCSs) genes and mutations in negative regulators of cytokine signalling, including Casitas B-cell lymphoma (CBL), have been identified as additional mutations involved in the JAK-STAT pathway[246,247].

Signalling proteins most often co-mutate with epigenetic regulators, suggesting that these two types of mutations might co-operate to drive the disease. Mutations in epigenetic modifiers include TET2, IDHI/2, DNMT3A, ASXL1 and EZH2[248]. Within this group, TET2 is the most commonly co-mutated gene with JAK2V617F, present in 12-37% of MPN cases[249] and 50% of chronic myelomonocytic leukaemia (CMML)[232].
ASXL1 mutations are present in 22% of MF patients and only 5% of PV and ET[248]. TET2 and IDH1/2 mutations have a frequency of 25% and 10% respectively in secondary AML while TP53 mutations have been observed in 46% of post-MPN AML[250].

1.2.4 MPN mouse models

The earliest efforts to model the molecular consequences of JAK2 V617F were undertaken using mouse models. In general, MPN mouse models faithfully re-capitulate the main aspects of human disease in mice, enabling the characterisation of disease-associated genetic alterations in the HSPC compartment, and providing *in vivo* models for testing novel therapeutic agents. Since JAK2V617F was the first driver mutation identified and is largely unique to MPNs, significant efforts have been made to establish mouse models expressing JAK2V617F, by using different genetic approaches.

More than 15 distinct JAK2V617F mouse models are comprehensively reviewed in Li et al.[251], including retroviral over-expression, transgenic, and targeted gene knock-in models. The most physiologically relevant models are the genetic knock-ins (KIs), where JAK2V617F is expressed under the control of endogenous elements. JAK2V617F KI models have been generated by a number of groups[252–255] and, although some differences in disease severity were observed, all models gave rise to a transplantable MPN-like disease.

In this thesis, I use the model generated by Li et al.[255,256], where a human JAK2V617F mutated cDNA is expressed under the control of the endogenous mouse *Jak2* promoter elements. Comparison between the heterozygous (JAK R/+, Het) and the homozygous (JAK R/R, Hom) JAK2V617F KI models shows a clear phenotypic switch between an ET-like and PV-like phenotype driven by the JAK2V617F homozygosity.

Het mice exhibited a raised platelet counts, increase in Hb resembling the human ET phenotype, while the Hom mice developed a PV-like phenotype, with striking erythrocytosis. A reduction in the frequency of HSPCs was observed in the model, as well as a progressive reduction in chimerism over time in both non-competitive and competitive transplantation settings. This model helped identify the effect of driver mutations on HSCs, showing that JAK2V617F mutation confers a proliferative advantage to progenitors and suggesting that additional factors are needed to drive a durable and robust MPN phenotype in the presence of non-mutant cells[257].

Up to 12% of MPN patients are associated with TET2 gene deletion or loss-of function mutations[258]. As TET2 is the most commonly co-mutated gene with JAK2V617F and also is commonly mutated in AML, a number of models for TET2 loss have been established[249,259,260]. All groups reported an increase in size of the HSPC compartment as well as a competitive transplantation advantage. TET2 KO mice have an increased number of myeloid cells, a decrease in erythroid and platelet counts and alteration in normal splenic architecture. With age, some TET2 KO mice developed a lethal disease characterised by anaemia, thrombocytopenia and high white cell counts, with differentiation abnormalities reminiscent of human myeloid disorders. The model we selected for our studies is the KO generated by Ko et al.[249].

Crosses between models of JAK2V617F and TET2 KO have been performed in order to better understand the interactions between these commonly co-mutated genes[261,262] and generally result in a more severe disease phenotype. In the model by Chen et al[261], TET2 loss in combination with the JAK2V617F mutation confers a functional competitive advantage to the HSCs, also resulting in a more complex MPN phenotype, when compared to that observed with either mutation alone. Mice had elevated haematocrit and platelets, and similar to the JAK2V617F mice, had increased HSC numbers in the spleen and increased splenomegaly and extramedullary haematopoiesis.

For this thesis, I used the JAK2V617F KI/ TET2 KO (JAK TET) cross from Shepherd et al.[262] and the CALR^{del52} mice from Li et al.[263]. The JAK TET mice show a robust serially transplantable MPN phenotype, with increased haematocrit and haemoglobin levels.

The heterozygous mice ($CALR^{del/+}$) develop an ET-like disease with marked thrombocytosis, while the homozygous mice ($CALR^{del/del}$) phenotype showed MF-like features, including reduced haematocrit, splenomegaly, leucocytosis and increased BM reticulin[263].

1.2.5 Moving beyond mutations in MPNs

Despite advances in the treatment of these disorders and substantial research into understanding the molecular network driving MPNs, a number of major unsolved questions exist, especially regarding the identification of high-risk patients and the use of predictive markers of disease evolution and leukemic transformation. Moreover, it remains unclear how a single genetic mutation, JAK2V617F, can associate with three different phenotypes, or why some patients eventually transform to more severe disease (5-20% post-PV/ET MF) or AML while some others remain stable. Recent studies have documented that JAK2V617F mutations can also be detected in normal individuals in the absence of any haematological disease[264–266]. Also confusing the picture is a small cohort of patients who lack a disease-causing genetic driver mutation (such as JAK2, CALR or MPL). This group is also named "triple-negatives" and represents 10-20% of MPN patients, with 12% of ET and 5% of pMF[267], reinforcing the idea that the genetic mutations cannot yet explain the whole disease and additional factors likely to contribute to driving distinct disease phenotypes.

A number of theories exist to explain disease heterogeneity and a number of factors have been proposed as possible contributors to the disease establishment and maintenance. The gene-dosage hypothesis suggests that the number of copies of JAK2V617F (hetero- or homozygous) is associated with different disease phenotype. Germline genetic factors have been proposed to contribute to the development of MPNs and to disease diversity. Having a first-degree relative with an MPN seems to increase the risk of developing the same disease by 5-7 fold[268]. Additionally, a specific haplotype block (46/1) increases the odds of developing an MPN by 3-4 fold[269]. A number of groups have also suggested that the presence of JAK2V617F co-mutated genes as well as the order they arise in, might contribute to MPN progression[238].

Importantly, in two independent KI models of MPNs, it was shown that the JAK2V617F mutation alone could generate a transplantable disease, although it failed to confer a significative competitive advantage over normal haematopoiesis, leading to the progressive exhaustion (or at least failure of outgrowth) of the mutant cell population[255,270,271]. This suggests that, despite the fact that JAK2V617F is clearly driving the disease phenotype, the mutant clone needs additional mutations (or some other factor) to acquire a relative clonal advantage in order for the disease to be established and/or maintained.

Increasing evidence is also emerging regarding the role of the microenvironment and whether it might contribute to the disease initiation or evolution: mutated haematopoietic cells can cause alterations in BM mesenchymal stem cells that contribute to the development of MPNs[272]. Moreover, it has been shown that BM cells isolated from MPN patients provide a better *in vitro* support to JAK2V617F-mutated $CD34^+$ cells (but not to the non-mutated $CD34^+$cells) than BM cells isolated from healthy donors[273], supporting the idea that mutated haematopoietic cells can remodel their niche into a more permissive environment that supports the mutated cells at the expense of the normal ones. This evidence highlights the potential contribution of the microenvironment to the disease heterogeneity of MPNs and its potential role in supporting the development and/or the maintenance of the disease. The next section will review the current state of the field in this area.

1.2.6 The cytokine microenvironment in MPNs

Considering that MPNs arise from HSCs and that MF patients have a high degree of BM fibrosis, it is reasonable to examine the impact of BM environment on disease and whether the signals received by cells might stimulate progression of malignancy, as previously observed in solid tumours[274,275]. However, studying the microenvironment of MPNs is challenging, largely due to the heterogeneous nature of the condition and the fact that MPNs often manifest at an older age, meaning there are a number of comorbidities present.

The first evidence that the niche could contribute to myeloid malignancies comes from transplantation experiments where an MPN-like disease arose from an altered

microenvironment, even in the absence of mutations in the haematopoietic cells themselves. Mib1 deletion causing dysregulated Notch signalling in non-haematopoietic cells can cause an MPN-like disease, which can be reverted[276]. Similarly, the retinoic acid receptor-γ (RAR-γ) and the retinoblastoma (Rb) protein can both induce a myeloid disease when constitutively deleted in non-haematopoietic cells[277], giving strong evidence that the microenvironment could contribute to myeloid diseases.

As overproduction of certain proinflammatory cytokines has been observed in MPN patients, most extensive work has been undertaken on specific soluble factors (e.g., TNF-α, IL-6, TGF-β). A straight example is TNF-α, whose plasma levels have been reported to be increased in plasma from MPN patients compared to healthy individuals, and such levels increase with increased disease severity (ET>PV>MF) and correlate with JAK2V617F burden[278]. A similar rise in TNF-α was observed in serum from mice with retrovirally induced JAK2V617F MPNs[279]. The correlation between JAK2V617F and TNF-α expression was further confirmed by cell line data, showing that HEL cells expressing JAK2V617F have the highest TNF-α expression. The TNF-α rich environment created by the JAK2V617F mutation also confers clonal selection for TNF-α resistant cells, supporting the idea that the environment contributes by applying a selective pressure for expansion of the malignant over the normal clones.
The pro-inflammatory cytokine Lipocalin-2 is elevated in serum from PV, ET, PMF and secondary MF (sMF). It stimulates the growth of BM cells derived from MF patients specifically but not those isolated from healthy donors and it is able to suppress normal haematopoiesis, by inducing DNA damage and apoptosis in normal but not in MF haematopoietic cells[280]. In both patients and MPN mouse models, megakaryocytes and monocytes have been shown to secrete a number of cytokines, including FGF, IL-8, TFG-β, and vascular endothelial growth factor (VEGF), which stimulate angiogenesis and drive fibroblast differentiation, leading to BM fibrosis[281,282]. Through the secretion of soluble factors, such as TPO, C-C ligand 3 (CCL3) and IL-1β, the mutant clone seems to be able to remodel the BM niche and to create a more permissive environment to facilitate clone expansion[283,284].

In the last decade, different clinical studies showed that some of the constitutional symptoms in MPN patients were concomitant with changes in the plasma levels of many cytokines[206,278,285–288]. Increased cytokine levels in serum from MPNs was first described more than 15 years ago, when different groups reported that alteration in serum levels of IL-6[206,289],IL-2[290], and TNF-α[291], were associated with blood cell count abnormalities. In a later study, Hermoeut et al. showed that concentrations of IL-11 and IL-8 were elevated in serum from PV patients[292]. Shortly thereafter, Panteli et al. conducted a clinical study on 73 MPN patients (25 PMF, 40 ET and 8 PV), measuring serum levels of IL-1α, IL-1β, IL-2, IL-6, soluble IL-2 receptor α (sIL2-Rα) and TPO[206]. The authors found that all three MPN subtypes had increased serum levels of IL-2 compared to healthy subjects. In addition, a significant increase in TPO serum levels was observed exclusively in pMF patients.

More recently, Tefferi et al. assessed plasma levels of 30 cytokines, by using a multiplex assay based on magnetic nanobeads coupled with flow cytometry[207]. This analysis confirmed the previously described raised levels of IL-2, IL-6, and IL-8 and found further significant differences in 20 of the 30 cytokines tested. Using the same multiplex assay technology, Vaidya et al. focused their study on profiling over 20 cytokines in a cohort of 192 pMF patients and 35 controls. Comparisons with healthy controls showed significantly higher levels of several cytokines. Levels of several cytokines were significantly higher in pMF but not in PV, showing numerous differences between PV and pMF profiles[285].

A study from Pourcelot et al. confirmed the previously reported rise in IL-6, IL-8, IL-12, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF) and VEGF in PV patients. Moreover, the authors showed that a number of cytokines were elevated in ET patients compared to PV[286] (Table 1).

Table 1. Clinical studies on serum levels in MPN patients.
Table lists all different clinical studies which reported alteration in cytokine levels in serum from MPN patients. Number of patients, MPN subtypes, number of cytokines analysed, and presence of longitudinal and *in vitro* functional studies are listed, highlighting the main gaps in the field. MPN=Myeloproliferative neoplasms, ET=essential thrombocythemia, PV=polycythaemia vera, MF=myelofibrosis, IL-6=Interleukin-6, IL-8=Interleukin-8, TNF-α=Tumour necrosis factor-α, HGF=Hepatocyte growth factor, IL-11=Interleukin-11.

	Number of patients	MPN subtypes	Number of cytokines	Longitudinal studies	Functional studies
Hermoeut et al. 2002[292]	n=18	PV only	n=2	-	-
Panteli et al. 2005[206]	n=73	ET, PV, MF	n=6	IL-6	-
Ho et al. 2007[293]	n=20	ET, PV, MF	n=79	-	-
Fleischmann et al. 2008[278]	n=48	ET, PV, MF	n=1	-	TNF-α
Tefferi et al. 2011[207]	n=127	MF only	n=30	IL-8	-
Boissinot et al. 2011[208]	n=122	ET, PV	n=7	-	HGF, IL-11
Vaidya et al. 2012[285]	n=192	PV, MF	n=20	-	-
Pourcelot et al. 2014[286]	n=38	ET, PV	n=13	-	-

From all the clinical studies, evidence has emerged that all the MPN subtypes are characterised by an important change in cytokine production, as seen from the observed increase in plasma levels of several inflammatory cytokines (such as IL-1, IL-2, IL-6, IL-8, IL-12, TNF-α, IFN-γ) and many growth factors (GM-CSF, G-CSF, HGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF)). Importantly, differences in levels of cytokine profile have been reported across the three MPN subtypes.

Taken together, these studies confirmed the existence of an inflammatory microenvironment that is associated with MPNs and the putative clinical impact of deregulation of cytokines in the diagnosis of haematopoietic disorders. The common variable shared between different disease cases is the atypical and prolonged cytokine production, which suggests that chronic inflammation might play an important role in MPN development and that disease progression may in part be derived from specific aspects of a patient's immune system.

However, it is difficult to compare prior cytokine screen results to each other since these studies have either focused on a small number of cytokines or a single subtype of disease (and very few included ET patients in their analysis) and none of them performed longitudinal analysis of large multi-disease patient cohort to study intra-

MPN differences. Moreover, few studies completed so far complemented the analysis with *in vitro* functional assays, in order to functional characterise the role of inflammatory factors in the context of the disease initiation and maintenance[208,278].

Additionally, it is still not clear to what extend cytokines contribute to the development and maintenance of MPNs and whether differences between patients reflect differences in MPN phenotype.

1.2.7 Clinical importance of cytokines in the treatment of myeloproliferative neoplasms

Since the discovery of specific gene mutations in MPNs[222,223,225–227] , the field has rapidly progressed with advanced treatment and diagnosis options. Available treatments include anti-inflammatory agents such as steroids, aspirin or immune-modulatory agents[228,235,294], which mainly aim at decreasing the number of certain blood cells and reducing the risk of complications. The choice of the type of treatment depends on the MPN subtype and/or the severity of the disease with no single treatment being effective for all MPN patients. On the other hand, subtype diagnosis is relatively inconsistent and recent molecular profiling data suggests that substantial heterogeneity can be resolved by including driver mutation profiling in disease classification/prognosis.

Following the large number of studies showing that dysregulation in cytokine levels associate with a number of haematological malignancies and leukaemia, several cytokine alterations have been specifically addressed in various experimental treatments. In addition, a large number of clinical trials have started exploring the efficacy of cytokine-based drugs, alone, or in combination with other immunomodulatory drugs, to treat human malignancies and cancers.

A human anti-TFG-β mono-clonal antibody (Fresolimumab) was used in a phase 1 study for the treatment of melanoma and renal-cell carcinoma (RCC)[295] and several other potential anti-TGF-β compounds which are in clinical development in cancer have recently been reviewed[296]. IL-2 also demonstrated clinical benefit and consequently received the approval for the treatment of metastatic renal cell carcinoma[297] and metastatic melanoma. Treatment with TNF-α antagonists have

shown therapeutic activity in mouse models and were used in phase 1 and 2 clinical cancer trials with some evidence of clinical activity[298].

A number of cytokines seem to be able to limit tumour cell growth by a direct pro-apoptotic or anti-proliferative activity, or by stimulating the cytotoxic activity of immune cells against the tumour. IFN-γ, as well as other cytokines such as IL-7, IL-12, IL-15 and IL-21, also gave promising results in immunotherapy based on pre-clinical animal models, but most of them are still being investigated clinically[299]. Since the first approval of IFN-α for the treatment of hairy cell leukaemia[300,301], the interferon family of cytokines has been used for the treatment of multiple haematological malignancies and solid tumours, including follicular non-Hodgkin lymphoma[302], melanoma[303], and AIDS-related Kaposi's sarcoma[304]. More recently, IFN-α2 has been revisited in patients with myeloproliferative neoplasms and with CML, in combination with anti-inflammatory agents, such as ruxolitinib[305].

Immune dysregulation and pro-inflammatory state are central feature of MPNs. Despite the fact that immunotherapy data in MPN patients are very limited and the validity of this approach remains to be defined[306], there is more evidence that targeting the inflammatory environment that can drive clonal evolution might reduce the risk of transformations into more severe disease or even to AML and lower the impact of the inflammatory niche on tumour development.

Aims of the project

Mimicking the complexity of the stem cell "niche" for *in vitro* HSC expansion has proved to be a challenging task, as niches are composed of multiple cell types with specialised properties, secreting specific soluble factors and providing physical interactions, which are essential for the retention of HSC function[307–311]. Later and more recent attempts to expand HSCs outside the body focus on stromal-free cultures and the use of haematopoietic cytokines, which have been proved to support HSCs *in vivo*. However, such cultures often require HSCs to be cultured for several days or weeks in rich cytokine media, leading to the majority of cells in any given culture being non-HSCs and losing their functional properties. In addition, not having robust *in vitro* purification strategies compromise molecular studies of factors necessary for HSC maintenance.

Overall, this thesis aims at assessing the impact of extrinsic regulators on HSC fate and explores their potential role in driving the evolution of clonal haematological malignancies.

Chapter 3 focused on trying to devise a system to study HSCs in the absence of their niche in order to resolve the essential molecular components of self-renewal *in vitro*. Keeping HSCs in minimally supportive medium resulted in us being able to culture them for extended periods in the absence of cell division with the full retention of functional properties, thereby allowing us to study a niche-independent HSC at the single cell level.

Chapter 4 and 5 aimed to understand how extrinsic regulators might contribute to disease development since previous studies had implicated environmental factors in pre-leukemic disorders such as MPNs. No prior study was comprehensive regarding number and disease subtype of patients, genetic background, or cytokines assessed. To address these issues, Chapters 4 of my thesis was aimed at identifying which inflammatory molecules might play a role in MPN disease pathogenesis and determining the relative risk of disease transformation. Chapter 5 followed up the

functional role of one such candidate molecule (IP-10) in both *in vitro* and *in vivo* assays in murine models of MPNs.

2 Materials and Methods

2.1 Mice

All mice were maintained in the Central Biomedical Service (CBS) animal facility of Cambridge University and were housed in specific pathogen-free environment according to institutional guidelines. C57BL/6-Ly5.2 (WT) were purchased from Charles River Laboratories (Saffron Walden, Essex, UK). C57BL/6$^{W41/W41}$-Ly5.1 (W41) were bred and maintained in the CBS animal facility. JAK2V617F KI mice[256] were crossed with *Tet2* KO mice[249]. Homozygous B6.129S4-Cxcl10tm1Adl/J (IP-10 KO) were purchased from The Jackson Laboratory[312]. JAK2V617F KI/IP-10 KO mutant mice were obtained by crossing JAK2V617F KI from Li et al.[256] with IP-10 KO mice from The Jackson Laboratory[312]. NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice were obtained from Charles River. Experimental cohorts consisted of female age-matched animals (12-16 weeks of age at the time of transplantation). All procedures were performed under project license PPL 70/8406 and PPL P846C00DB, in compliance with the guidance on the operation of ASPA (Animals Scientific Procedures Act 1986), following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

2.2 Isolation of mouse ESLAM HSCs

BM cells were isolated from spine, sternum, femora, tibiae and pelvic bones of both hind legs of WT mice, by crushing the bones in 2% Foetal Calf Serum (FCS, STEMCELL or Sigma Aldrich, St. Louis, MO, USA (Sigma)) in PBS (Sigma). Red cell lysis was performed by treatment with Ammonium Chloride (NH_4Cl, STEMCELL). Depletion of mature lineage cells was performed using EasySep mouse haematopoietic progenitor cell enrichment kit (STEMCELL). HSCs were isolated from the lineage depleted cell suspension by using fluorescence-activated cell sorting using CD45$^+$EPCR$^+$CD48$^-$CD150$^+$Sca-1^{high} (ESLAM Sca-1^{high}) as described previously[313] (Figure 6), using CD45 FITC (clone 30-F1,1 BD Biosciences, San Jose CA, USA (BD)), EPCR PE (clone RMEPCR1560, STEMCELL), CD150 PE- Cy7 (clone TC15-12F12.2, both from Biolegend, San Diego, USA (Biolegend)), CD48 APC (clone HM48-1, Biolegend), Sca-1

Brilliant Violet (BV) 421 (clone D7, Biolegend) and 7- Aminoactinomycin D (7AAD) (Life Technologies, Carlsbad, CA, USA (Life Technologies)).

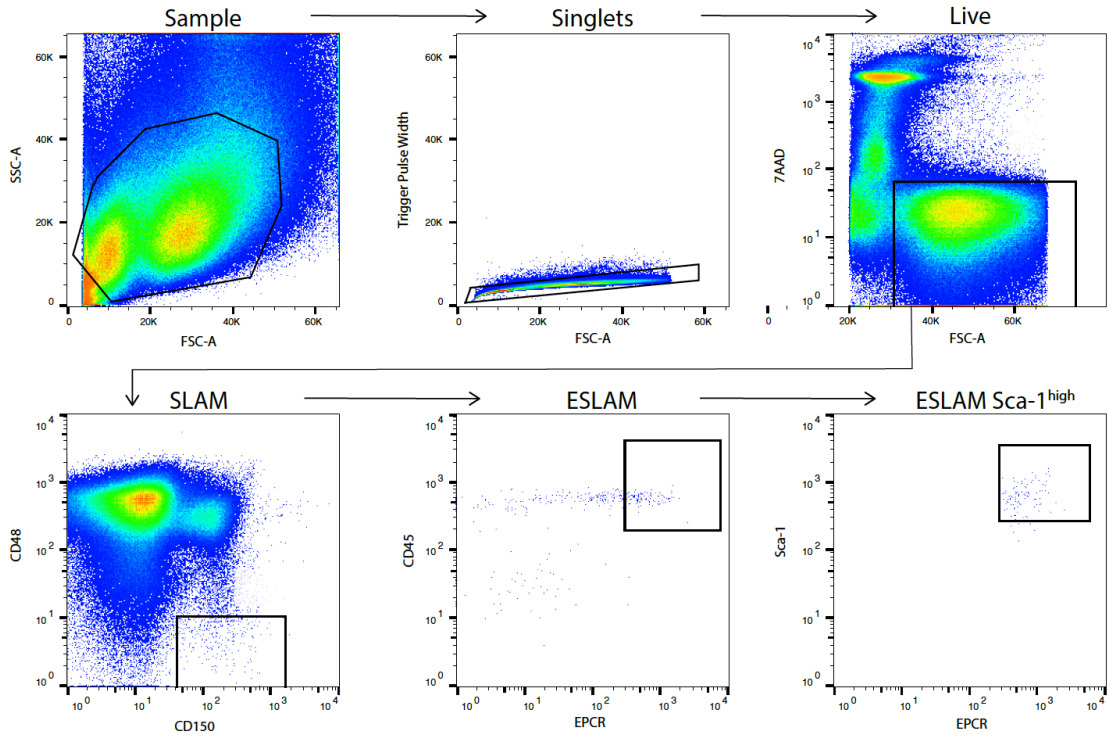


Figure 6. Representative gating layout to purify ESLAM Sca-1^{high} LT-HSCs.
CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca-1^{high} (ESLAM Sca-1^{high}) LT-HSCs were isolated from lineage-depleted BM by fluorescence assisted cell sorting.

The cells were sorted in either purity or single sort mode on an Influx cell sorter (BD) using the following filter sets 488 530/40 (for FITC), 561 585/29 (for PE), 405 460/50 (for BV421), 640 670/30 (for APC), 561 750LP (for PE/Cy7), 640 750LP (for APC/Cy7), 405 520/35 (for BV510), and 561 670/30 (for 7AAD). When single HSCs were required, the single-cell deposition unit of the sorter was used to deposit 1 cell into each well of a round bottom 96-well plate, each well having been preloaded with 50-100uL of medium.

2.3 Liquid culture and clone size determination of mouse HSCs

Single HSCs were sorted into 96-well U-bottom plates (Corning) and cultured in 100µL StemSpan SFEM (STEMCELL) supplemented with 100 units/mL Penicillin and 100µg/mL Streptomycin (Pen/Strep, Sigma), 2mM L-Glutamine (Sigma), 10^{-4}M 2-

Mercaptoethanol (Sigma) and 20ng/mL IL-11 (Biotechne, Abingdon, UK (Biotechne)), 300ng/mL Stem Cell Factor (SCF, R&D). 50 ng/mL IP-10 (Miltenyi Biotec, USA) was supplemented when specified. 10% of FCS was supplemented when stated. For serum-free cultures, cells were sorted into Ham's F12 nutrient mixture (Gibco, ThermoFisher, Waltham, MA, USA (Gibco)) supplemented with 20 ng/mL human IL-11 (R&D), 300 ng/mL SCF (SCT or R&D), 2 mM L-Glutamine (Sigma), 1000 U/mL-100 µg/mL Penicillin-Streptomycin (Sigma), 1% ITS-X (Insulin-Transferrin-Selenium-Ethanolamine, Gibco), 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Sigma), 100 mg/mL human serum albumin (HSA, Albumin Bioscience, Huntsville, AL, USA).

Cells were cultured at 37°C, 5% CO_2. Cell counts were performed daily every 22-24 hours and cell cycle kinetics determined for the first and second division by visual inspection, manually scoring each well as having 1, 2, or 3-4 cells. Cell survival was assessed by visual inspection on day 10 (the sorting day is determined as day 0) and clones were scored as very small (VS, less than 50 cells), small (S, 50-500 cells), medium (M, 501-5,000 cells), large (L, 5,001-10,000), and extra-large (XL, 10,001 or more cells) (Figure 7).

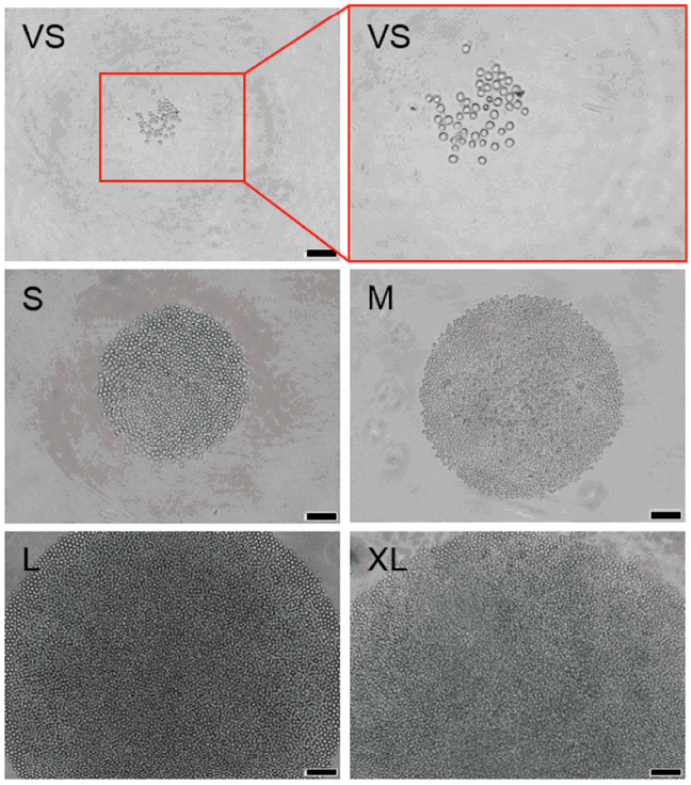


Figure 7. Representative images of colony sizes in liquid culture on day 10.
Single CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca-1^{high} (ESLAM Sca-1^{high}) LT-HSCs were cultured *in vitro* for 10 days. On day 10, clone size was assessed for survival and clone size by visual inspection. Scale bar represents 100µm. VS=very small, S=small, M=medium, L=large, XL=extra-large. Figure taken from Oedekoven[314].

2.4 Time lapse of single mouse HSCs

Single cells were sorted into 96-well U-bottom plate (Corning) and imaged on a Leica DMI3000 B microscope, housed inside an Okolab CO_2 microscope cage incubator system. Custom written LabVIEW software was used to control a Prior Proscan III nanopositioning stage and acquire images via a Hamamatsu Orca Flash 4.0 camera. Cells were imaged every 50 minutes for the first 7 days, the fastest time resolution achievable with the system while allowing enough time for the autofocus routine to correctly execute at all 96 wells. On day 7, the plate was removed and 300ng/mL SCF (SCT or R&D) was added to the 67 wells where there was a possibility of a viable cell, determined by eye. The reduction in well number allowed for an increase in time resolution to 35 minutes. By day 11, imaged well number was further reduced to 17 wells as it became more apparent in which wells cells were still viable. This allowed for a corresponding increase in time resolution to 20 minutes. Imaging continued until day 14.

2.5 Colony-forming assay of mouse HSCs

Single cultured cells (hibernated HSCs) were transferred from liquid culture into 600μL of MethoCult GF3434 (STEMCELL). Freshly isolated HSCs were isolated by FACS sorting (as described above) and plated into 3mL MethoCult GF3434 (STEMCELL) and split across 2 wells of a 6-well plate (STEMCELL). Cells were cultured for 14 days, colony numbers assessed by visual inspection and colony type scored by antibody staining with CD41 FITC (clone MWReg30) for the detection of megakaryocyte/platelet lineage, Ter-119 PE-Cy7 (clone TER-119) for erythrocyte lineage, CD45.2 APC- Cy7 (clone 104) for detection of haematopoietic cells, Ly6g Gr1 BV421 (clone 1A8) for granulocytes, CD11b Mac1 APC (clone M1/70) for monocytes/granulocytes. All antibodies were obtained from Biolegend. Samples were acquired on LSR II Fortessa (BD) and flow cytometry data analysed using FlowJo (Treestar, Ashland, OR, USA). Colonies were classified into: MK (containing cells positive for megakaryocyte marker $CD41^{+}$), GM (containing cells positive for granulocyte/monocyte markers Gr1 and Cd11b), GEM (positive for GM and erythrocyte markers Gr1, Cd11b, and Ter-119), GMM (positive for GM and MK markers), and GEMM (positive for GM, MK and E markers) colonies.

2.6 Bone marrow transplantation assay

Donor cells were obtained from WT mice (CD45.2) between 6 and 16 weeks of age. Recipient W41 mice were sub-lethally irradiated with a single dose (400cGy) of Caesium irradiation and all transplantations were performed by intravenous tail vein injection using a 29.5-G insulin syringe. For single cell transplantations, single HSCs were deposited by FACS into 100μL of medium in a 96-well U-bottom plate (Corning). All liquid was subsequently mixed with extra 100-400μL of PBS and aspirated into the insulin syringe (avoiding air bubbles) and injected into the tail vein. For secondary transplantations, whole BM was obtained from primary recipients by flushing tibiae and femurs with 2%FCS in PBS. Red cell lysis was performed and an equivalent of one femur of each donor mouse (~2 x 10^7 cells) was transplanted into at least two secondary recipients.

2.7 Lentiviral transduction of HSCs

7000 ESLAM HSCs cells were isolated and split between 4 wells (1750 cells/well) of a 96-well plate (Corning). Following their isolation, cells were kept in 50 μL of medium (StemSpan, 10%FCS, 20ng/mL IL-11) and were supplemented with polybrene (Sigma) and pHIV-ZsGreen CSTVR lentivirus supplied by Dr Alasdair Russell from Cancer Research UK (CRUK). Plates were centrifuged at 600g for 30 minutes, at 30°C, to promote infection, before being transferred into a 37°C incubator. Two days after, cells were collected from the wells and resorted for viability ($7AAD^-$). Live cells (4001) were transplanted into 6 sub-lethally irradiated CD45.1 W41 recipient mice (for an approximate dose of 615 cells/mouse) and monitored for donor chimerism as described above, and GFP expression.

2.8 Single cell RNA sequencing analysis

Single cell RNA sequencing (scRNA-seq) analysis was performed as described previously in Picelli et al. 2014[315] (Smart-seq2). Single ESLAM HSCs were sorted by FACS into 96-well PCR plates containing lysis buffer (0.2% Triton X-100 (Sigma), RNase inhibitor (SUPERase, Thermofisher), nuclease-free water (Thermo Fisher)) Illumina Nextera XT DNA preparation kit was used to prepare the libraries, which were pooled and run on the Illumina Hi-Seq4000 at the CRUK Cambridge Institute Genomics Core. Cells from which low-quality libraries with insufficient sequencing depths were excluded by setting the threshold of number of mapped reads to >2*10, with mapped reads comprising nuclear genes, mitochondrial genes and ERCCs. A minimum threshold of 20% for reads mapping to known genes was set, in order to exclude empty wells and dead cells. In addition, the threshold for reads mapping to mitochondrial genes was >0.2, to ensure a minimum of 20% of reads to map to non-mitochondrial genes. Protein-coding genes were extracted for further processing.

2.9 Analysis of single-cell RNA sequencing

Computational analyses were performed in the R programming environment (version 3.6.3)[316,317]. Raw data was processed using the Seurat tool (version 3.2.0) The recommended standard processing pipeline was applied to perform log-normalisation (default settings) and identify highly variable genes

(nfeatures=10,000). Subsequently, expression values were scaled using default parameters. Dimensionality reduction, including principal component analysis (PCA) and Uniform Manifold Approximation and Projection (UMAP) was performed using default Seurat tools. Differential gene expression was performed using negative binomial generalised linear models, as implemented by DESeq2 (version 1.26.0)[318]. Genes with adjusted p-value <0.05 and logFC >1.5 were considered significantly differentially expressed (Benjamini-Hochberg corrected). Cell cycle scoring was performed based on average expression of key cell cycle genes, as described previously[319]. Similarly, gene set scoring was computed for previously described HSC proliferation and quiescence signatures[320]. Batch effect testing and correction was performed to inform any potential influence of technical bias. Normalisation and variable gene scoring were computed for each batch separately, using variance stabilising transformation. Subsequently, separate batches were integrated using canonical correlation analysis (CCA) by computing integration anchors (parameters: dims = 1:30 and k.filter = 10)[316]. A very limited batch correction was identified between day 1 and day 2 batches. However, full data integration introduced extensive over-correction and downstream analysis was performed without batch correction. All data visualisation was computed in R.

To compute gene ontology (GO) and KEGG pathway enrichment, gene symbols were converted to Entrez gene identifiers, using the mouse genome annotation database (org.Mm.eg.db, version 3.10.0). GO terms were extracted from the GO annotation database (GO.db, version 3.10.0). GO term enrichment and KEGG pathways analysis was computed using the Limma package (version 3.42.2). An adjusted *p-value* < 0.05 cutoff was set to determine GO term or KEGG pathway enrichment. Genes identified as significantly differentially expressed between cell types were used conduct pathway enrichment. Gene set enrichment analysis (GSEA) was performed using the UC San Diego-Broad Institute GSEA software (version 4.0.3)[321,322]. Pre-ranked gene lists were computed based on differentially expressed genes. GSEA was computed using multiple databases, including GO biological processes, KEGG pathways and the Reactome database. Analysis parameter were set as follows: 1000 permutations, weighted enrichment, minimum 15 and maximum 500 genes annotated to gene set.

2.10 Isolation of human cord blood and peripheral blood LT-HSCs

CB samples and PB samples were obtained from Cambridge Blood and Stem Cell Biobank (CBSB) with informed consent from healthy donors, in accordance with regulated procedures approved by the local Research and Ethics Committees. Mononuclear cells (MNCs) were isolated using Lymphoprep (Axis Shield PLC, Dundee, UK) or Pancoll lymphocyte separating medium (Pancoll, PAN Biotech, Aidenbach, Germany). Blood was mixed with equal volume of PBS and layered on Lymphoprep/Pancoll. Layered blood was centrifuged at 1400rpm for 25 minutes, at room temperature with the brake off. The MNC layer was carefully aspirated and washed with PBS, to remove any separating medium trace. Red cell lysis was subsequently performed by using red cell lysis buffer (Biolegend, San Diego, CA, USA (Biolegend)). CB cells were depleted of differentiated haematopoietic cells by using the human CD34 microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with the following modifications: all cells were resuspended in 90 μL 2%FCS in PBS per 10^8 cells, CD34 Microbeads were used at 30 μL/10^8 cells and FcR Blocking Reagents at 30μL/10^8 cells and FcR Blocking Reagents at 30μL/10^8 cells. Cells were separated using the AutoMACS cell separation technology (Miltenyi Biotec). PB cells were depleted using enriched for $CD34^+$ cells (EasySep Human CD34+ enrichment kit (STEMCELL) as per the manufacturer's guidelines except that only one round of depletion in the magnet was performed.

CD34 enriched cells were stained with CD34 PE-Cy7 (clone 581, Biolegend), CD38 FITC (clone HIT2, Biolegend), CD45RA V450 (clone HI100, Biolegend), CD90 APC (clone 5E10, Biolegend), CD3 APC-Cy7 (clone HIT3A, Biolegend), CD19 PE (clone HIB19, Biolegend) and 7AAD (Life Technologies) was used as a cell viability marker. HSCs were sorted as $CD34^+$, $CD38^-$, $CD90^+$, $CD45RA^-$, $CD3^-$, $CD19^-$ (Figure 8), on a FACS Aria fusion sorter (BD) at the NIHR Cambridge BRC Cell Phenotyping Hub facility or an Influx sorter (BD) at the Cambridge Research UK. The cells were sorted in either purity or single sort mode, using the following filter sets: 530/40 (for FITC), 750LP (for APC-Cy7 and PE-Cy7), 670/30 (for APC and 7AAD), 460/50 (for Violet450), and 585/29 (for PE).

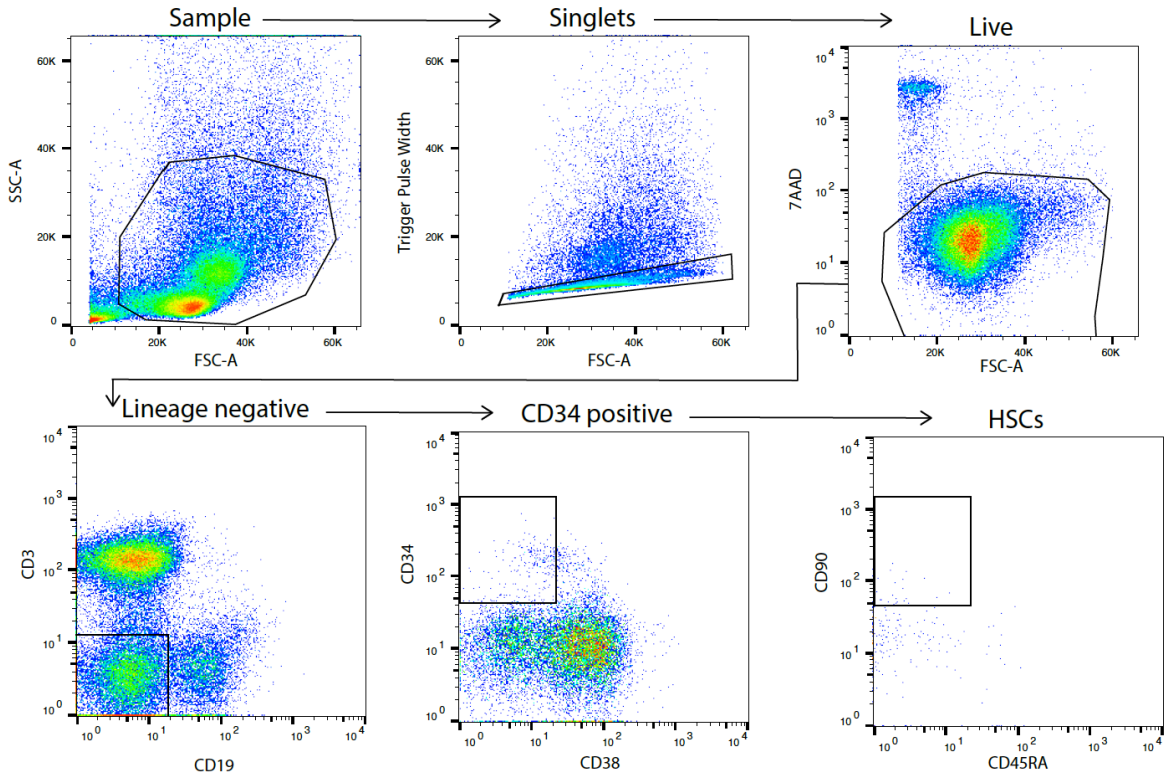


Figure 8. Representative gating layout for the isolation of human HSCs from peripheral blood and cord blood samples.

Single cells were sorted into individual wells of a 96-well U-bottom plate (Corning), each well having been preloaded with 50-100µL medium.

2.11 Liquid cultures and clone size determination of human LT-HSCs

Single HSCs were sorted into 96-well U-bottom plates (Corning) and cultured in 100µL StemSpan SFEM (STEMCELL) supplemented with 100 units/mL Penicillin and 100µg/mL Streptomycin (Pen/Strep, Sigma), 2mM L-Glutamine (Sigma), 10^{-4}M 2-Mercaptoethanol (Sigma) and 20 ng/mL IL-11 (Biotechne, Abingdon, UK (Biotechne)), 300ng/mL Stem Cell Factor (SCF, R&D) (added when specified), 10% FCS (added when specified). Cell survival was assessed by visual inspection on day 10 (the sorting day is determined as day 0).

2.12 Xenotransplantation

10,862 LT-HSCs were sorted and cultured into a single well (96-well U-bottom plate (Corning)) and cultured for 7 days as described above for the single cell culture. On

day 7, cell number was assessed by visual inspection and cells were serially diluted in PBS as following: ~110 cells split into 5 recipients (~22 cell per mouse), ~440 cells split into 4 recipients (~110 cells per mouse), ~654 cells split into 3 recipients (~218 cells per mouse). NSG mice were sub-lethally irradiated with a single dose (2.4Gy) by Caesium irradiation. Twenty-four hours later mice were anaesthetised with isoflurane and injected intrafemorally as previously described[21].

2.13 Peripheral Blood Analysis

PB samples were collected in EDTA coated microvette tubes (Sarstedt AGF & Co, Nuembrecht, Germany). Blood was collected from the tail vein at week 8, 12, 16, 20 post-transplantation, unless otherwise stated. Red cell lysis was performed by using NH_4Cl (STEMCELL) and samples were subsequently analysed for repopulation levels as previously described[20,33]. Cells were stained for lineage markers using Ly6g BV421 (clone 1A8), B220 APC (clone RA3-6B2), CD3 PE (clone 17A2), CD11b (or Mac-1) PE-Cy7 (clone M1/70), CD45.1/AF700 (clone A20), CD45.2 FITC (clone 104). All antibodies were obtained from Biolegend. 7AAD (Life Technologies) was used as a viability dye. Representative gating strategies are shown in Figure 9. After gating for singlets and viable, donor and recipient cells were distinguished based on CD45.1 (recipient) and CD45.2 (donor) expression. B cells were defined as viable single cells, myeloid negative (Mac-1^-, Ly6g$^-$), CD3^-, B220^+. T cells were defined as viable single cells, myeloid negative (Mac-1^+, Ly6g$^-$), B220^-, CD3^+. Cells of the myeloid lineage (GM, Granulocyte/Monocyte) were defined as viable single cells, lymphoid negative (B220^-, CD3^-), Mac1^+cells (Figure 9). Recipients with at least 1% donor white blood cells (WBCs) at 16-20 weeks after transplantation were considered to be repopulated. Recipients with at least 1% contribution to all three lineages are considered multi-lineage.

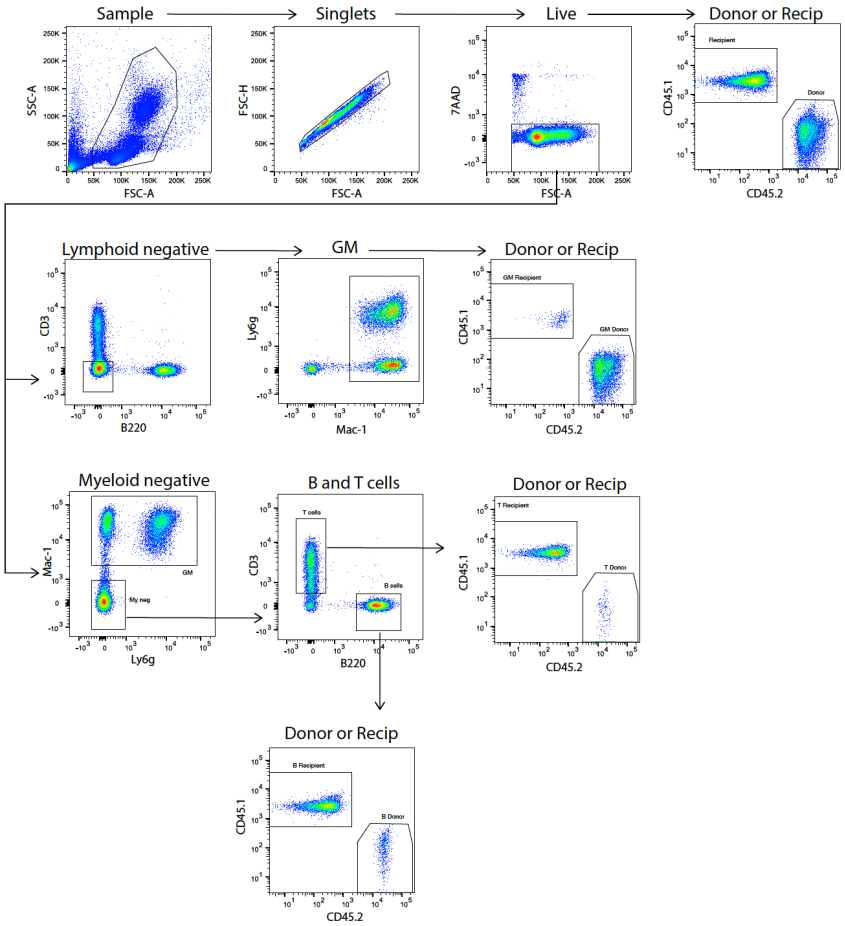


Figure 9. Representative gating layout for peripheral blood chimerism analysis.

Samples were acquired on LSR Fortessa (BD) and flow cytometry data were analysed by using FlowJo v10 (FLOWJO LLC, Ashland, OR, USA). Peripheral blood cell counts were performed using a Woodley ABC blood counter (Woodley Equipment, Bolton, UK).

For NSG mice, peripheral blood samples were collected in EDTA coated microvette tubes (Sarstedt AGF & Co, Nuembrecht, Germany). Blood (~100µL) was collected from the tail vein at 12- and 20-week post-transplantation. Cells were stained as following. Blood was transferred into polystyrene tubes (Becton Dickinson) and diluted 1:1 with 2%FCS in PBS. 1 mL of Lymphoprep (STEMCELL) was carefully layered at the bottom of the tube and the tubes were centrifuge for 25 min at 500g (brake off). MNCs were collected, washed with PBS and resuspended in 50µL of PBS/FCS and transferred into a 96 U-bottom plate (Corning) to stain. Cells were stained with the following lineage

markers: CD19 FITC (clone HIB19, Biolegend), GlyA PE (clone HIR2, BD) CD45 PE-Cy5 (clone HI30, Biolegend), CD14 PE-Cy7 (clone M5E2, Biolegend), CD33 APC (clone P67.6, BD), CD19 AF700 (clone HIB19, Biolegend), CD3 APC-Cy7 (clone HIT3a, Biolegend), CD45 BV510 (clone HI30, Biolegend). Samples were acquired on LSR Fortessa (BD) and flow cytometry data were analysed by using FlowJo v10 (FLOWJO LLC, Ashland, OR, USA). To detect human engraftment, two different antibodies against CD45 were used, and cells were considered human if positive for both ($CD45^{++}$) (Figure 10). Mice were considered successfully repopulated if the percentage of ($CD45^{++}$) ≥ 0.01% (and at least 30 cells were recorded in these gates).

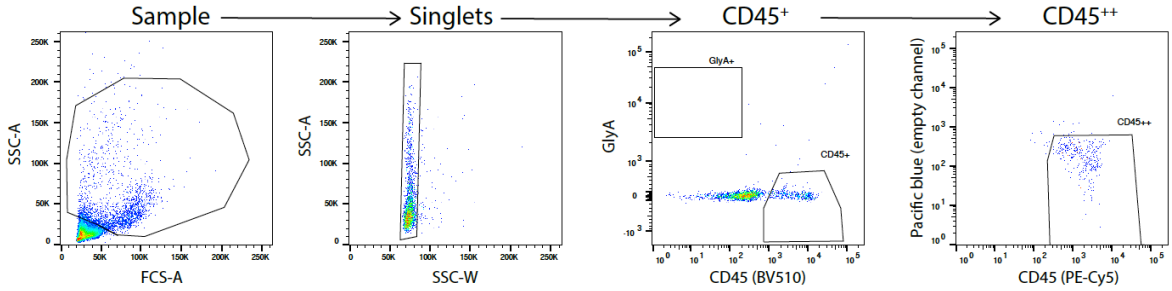


Figure 10. Representative gating layout for peripheral blood chimerism analysis of NSG mice transplanted with human HSCs.

2.14 Primary MPN patient samples

The studies included in this thesis included two groups of Philadelphia chromosome negative MPN patients: 1) main cohort (n=291 patients) and 2) a selected PT-1 cohort (n=122 patients) enriched for MF and AML transformation events. Patients from both groups were diagnosed according to British Committee for Standards in Haematology (BCSH) guidelines[323]. PB, serum, and BM samples were obtained in accordance with the Declaration of Helsinki, under the approval of the Cambridge and Eastern Region Ethics Committee or as part of the UK Medical Research Council PT-1 trial[324–327]. Samples were taken at the time of the initial patient visit on referral to the specialist MPN clinic. For the PT-1 patient cohort, 36% of patients had their samples collected before receiving any cytoreductive therapy and >80% of patients were sampled within the first 30 days. Targeted sequencing of the coding regions of 33 recurrently mutated genes was available for 239 patients (n=117 from Cambridge cohort, n=122 from PT-1 cohort)[328]. Longitudinal studies included repeat samples from UK patient

cohorts (n=81). Cytoreductive treatment information was available in PT-1 cohort patients and included in multivariate analysis. Baseline BM fibrosis data was available for a subset of patients (n=44). Historic frozen serum samples were obtained through CBSB and fresh PB samples were acquired through the Cambridge Blood and Transplant Centre.

2.15 Patient serum cytokine profiling

Fresh PB (40-60 mL) was collected in S-Monovette Z Gel clot activator tubes (Sarstedt) or BD Vacutainer tubes. After clot formation and centrifugation, serum aliquots were stored at -80°C or in liquid nitrogen (Cambridge Blood Stem Cell Biobank). Cytokine levels in patient serum samples and normal controls were assayed using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (Millipore). An initial 38-plex panel (Milliplex HCYTOMAG-60K-PX38) was run on 185 MPN patients and 14 healthy controls. The levels of 38 inflammatory cytokines and chemokines were measured: soluble forms of CD40 ligands (sCD40L), EGF, basic fibroblast growth factor (FGF-2), FLT-3L, Fractalkine, G-CSF, GM-CSF, growth-regulated oncogene-alpha (GRO-α, or CXCL1), IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17 (or IL-17A), interferon-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), MCP-3, macrophage-derived chemokine (MDC or CCL22), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, RANTES, TGF-α, TNF-α, TNF-β, VEGF, Eotaxin, PDGF-AA. A 10-plex custom assay was designed to profile a further 106 MPN cases (total=291), as well as an additional 122 ET patients for biomarker validation (PT-1 cohort). The levels of 10 inflammatory cytokines were measured: IP-10, IL-8, EGF, Eotaxin, TGF-α, IFN-γ, GRO-α, IL1-RA, TNF-α, IL-10. Each immunoassay was completed using 25μL serum (undiluted). Briefly, all reagents were equilibrated at room temperature (RT, 20-25 °C) and the supplied 96-well plate was loaded with 200μL of Wash Buffer and put in agitation for 10 minutes at RT. After removing the assay buffer by tapping it out, 25μL of the assay Buffer were added to the sample and background wells followed by 25μL of Matrix solution to background, standards and control wells. After thawing, 25μL of each patient serum sample were added to the sample wells. Pre-sonicated beads were mixed and added to each well and the plate was incubated overnight at 4°C (or 2 hours

at RT). After incubation, serum was removed by placing the plate on a magnet and tapping it out and the plate was then washed twice. 25µL of Detection Antibody was added to each well followed by 1-hour incubation at RT with agitation. 25µL of Streptavidin-Phycoerythrin was added to each well followed by 30 minutes incubation at RT. The plate was washed twice and 150µL of Sheath Fluid was added. To re-suspend the beads were using a plate shaker for 5 minutes. The plate was run on Luminex xMAP machine to detect MFI values for each analyte specific bead set. Cytokine concentrations were determined by xPONENT software (Luminex) based on the fit of a standard curve for MFI VS pg/mL. Used values derived from the known reference concentrations supplied by the manufacturer. All cytokines ranged within the quality control values. Raw cell values were exported from Magpix xMAP exponent software into Microsoft excel with pg/mL values calculated on the basis of the standard curves automatically generated. GRO-α levels exceeding a concentration of 10,000 pg/mL were depicted at a maximum value of 10,000 to ensure compatibility between samples.

2.16 *In vitro* culture of HSCs from MPN patients

Single HSCs from MPN patients or healthy donors' PB were sorted into 96-well U-bottom plates (Corning) and cultured in 100µL StemSpan SFEM (STEMCELL) supplemented with 100 units/mL Penicillin and 100µg/mL Streptomycin (Pen/Strep, Sigma), 2mM L-Glutamine (Sigma), 10^{-4}M 2-Mercaptoethanol (Sigma), cc100 cytokine cocktail (STEMCELL), with or without 50 ng/mL IP-10 (Miltenyi Biotec, USA). Cell counts were performed every 24 hours and cell cycle kinetics determined for the first and second division by visual inspection, manually scoring each well as having 1, 2, or 3-4 cells.

2.17 Mouse serum cytokine profiling

PB samples (≈150ul) were collected from the tail vein of homozygous JAK2V617F KI mice[256], TET2 KO mice[249], combinatorial JAK TET mice[262], CALR Del52 (CALR)[263], aged 16 weeks or by cardiac puncture for JAK Hom mice, due to phenotype reducing the amount of serum obtained per mL of blood. Samples were left undisturbed at RT for 30 minutes. The clot was removed by centrifuging the samples at 2,000x g for 10

minutes in a refrigerated centrifuge. The resulting supernatant (serum) was transferred into clean polypropylene tubes and subsequently stored at -20°C. Cytokine levels in mice serum samples were assayed using the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore). The levels of 32 cytokines were quantified: Eotaxin, GM-CSF, IL-1α, IL-2, IL-4, IL-6, IL-9, IL-12 (p40), IL-13, IL-17, VEGF, granulocyte-colony stimulating factor (G-CSF), interferon gamma (IFN-γ), IL-1β, IL-3, IL-5, IL-7, IL-10, IL-12 (p70), IL-15, IP-10, RANTES, KC, LIX, macrophage-colony stimulating factor (M-CSF), MIP-1α, MIP-2, TNF-α, leukaemia inhibitor factor (LIF), MCP-1, monokine induced by interferon-gamma (MIG), and MIP-1β. The plate was run on Luminex xMAP machine to detect MFI values for each analyte specific bead set. Cytokine concentrations were determined as listed above.

2.18 Genotyping of IP-10 KO mouse models

Ear biopsies were obtained from individual mice and placed in individual Eppendorf tubes. DNA extraction was performed as follow: 50μL alkaline lysis buffer (24.4mM NaOH (Sigma) and 195μM disodium EDTA (Life Technologies, Carlsbad, CA, USA) in water) was added and the tubes were incubated at 95°C, 1000rpm for 20 min, on a heated shaker. 12.5μL of Kapa2G master mix (Kapa biosystems), 10.9μL of nuclease free water and 0.6μL of 10μM of primers (common primer, and WT or Mutant, Table 2) were added to 1μL per sample. PCR products were run on 1% agarose gel (Biorad), using 1:10000 GelRed Nucleic Acid Gel Stain (Biotum) to visualize the PCR products.

Table 2. Primer sequences for IP-10 genotyping.

Primer	Sequence 5' → 3'
IP-10 WT	TGC CAC GAT GAA AAA GAA TG
IP-10 Mutant	GGG ACA GAA TGG GTT TGT GT
Common	GCC AGA GGC CAC TTG TGT AG

A representative image of a result is shown in Figure 48.

2.19 Transplantation into IP-10 KO recipients

Donor cells were obtained from JAK (Hom) mice or from JAK TET mice. All competitor cells were obtained from IP-10 KO mice and $3x10^5$ cells whole BM cells were transplanted alongside the donor cells (25/recipient). Recipients were WT, purchased from Charles River Laboratories (Saffron Walden, Essex, UK) or IP-10 KO

mice purchased by The Jackson Laboratory, irradiated with 2 doses (2x550Gy, separated by >4 hours), by Caesium irradiation and transplants were performed by intravenous tail vein injection using a 29.5-G insulin syringe.

2.20 Flow cytometric analysis of bone marrow and spleen from JAK IP-10 mice

BM and spleen tissues were collected from 16-week-old mice. As above, BM was processed by flushing tibiae and femurs with 2%FCS in PBS. Spleen was processed by gently cutting it into small pieces and then pressed through a 70-micron cell strainer (Greiner Bio-One Ltd) by using a 1mL syringe plunger.

After gating for viable, B- and T-cells populations were distinguished based on CD3 (T-cells) and B220 (B-cells) expression, GM population based on CD11b and Ly6g expression. The CD71/Ter-119 staining pattern define different stage of erythroblast differentiation (I through V) (Figure 11). The precise borders between these regions were determined arbitrarily[329].

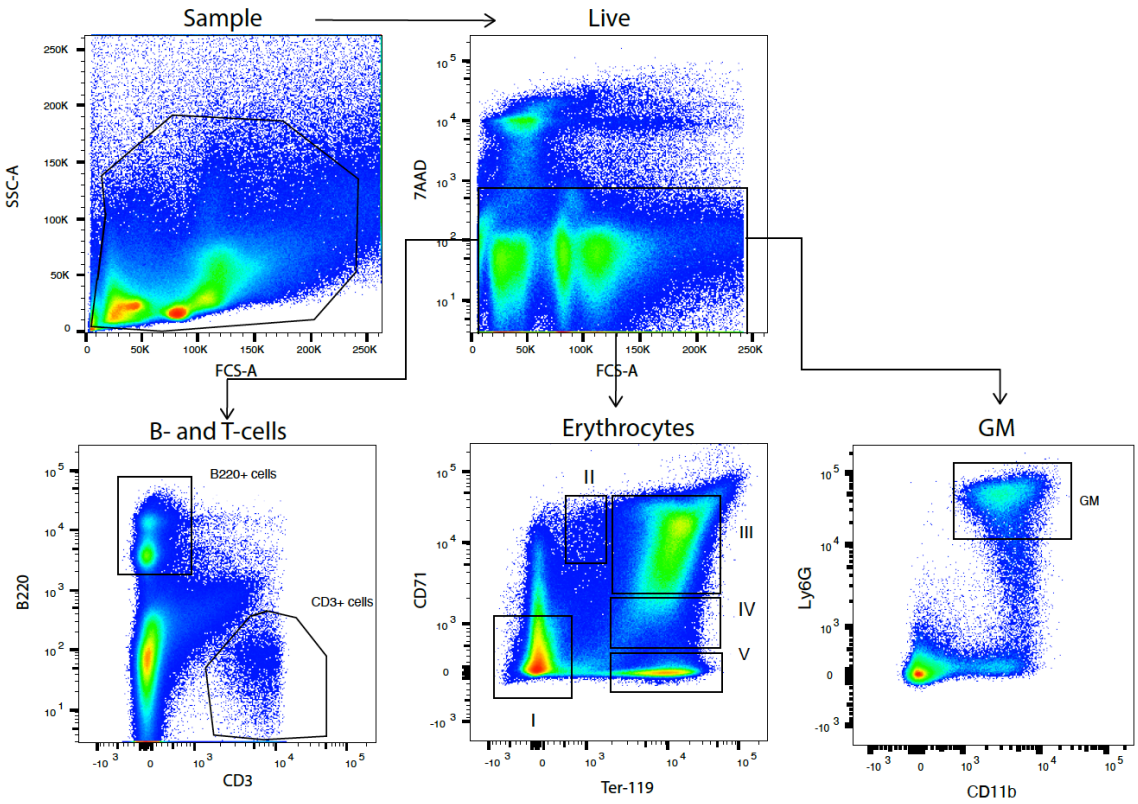


Figure 11. Representative gating strategy for BM phenotyping of B-cell, T-cell, GM, and erythroid populations.
B- and T-cell populations were discriminated based on B220 and CD3 expression respectively, GM were defined as CD11b^{+}, Ly6g^{+}. CD71 and Ter-119 profiles define progressive stages of erythroid differentiation (I through V).

For LSK analysis, BM and spleen cells were stained using Sca-1 BV421 (Clone D7), c-kit APC-Cy7 (clone 2B8), Lin BV510 (Figure 12). All antibodies were obtained from Biolegend. LSK were defined as single viable Lin^{-}c-kit^{+}Sca-1^{+} cells.

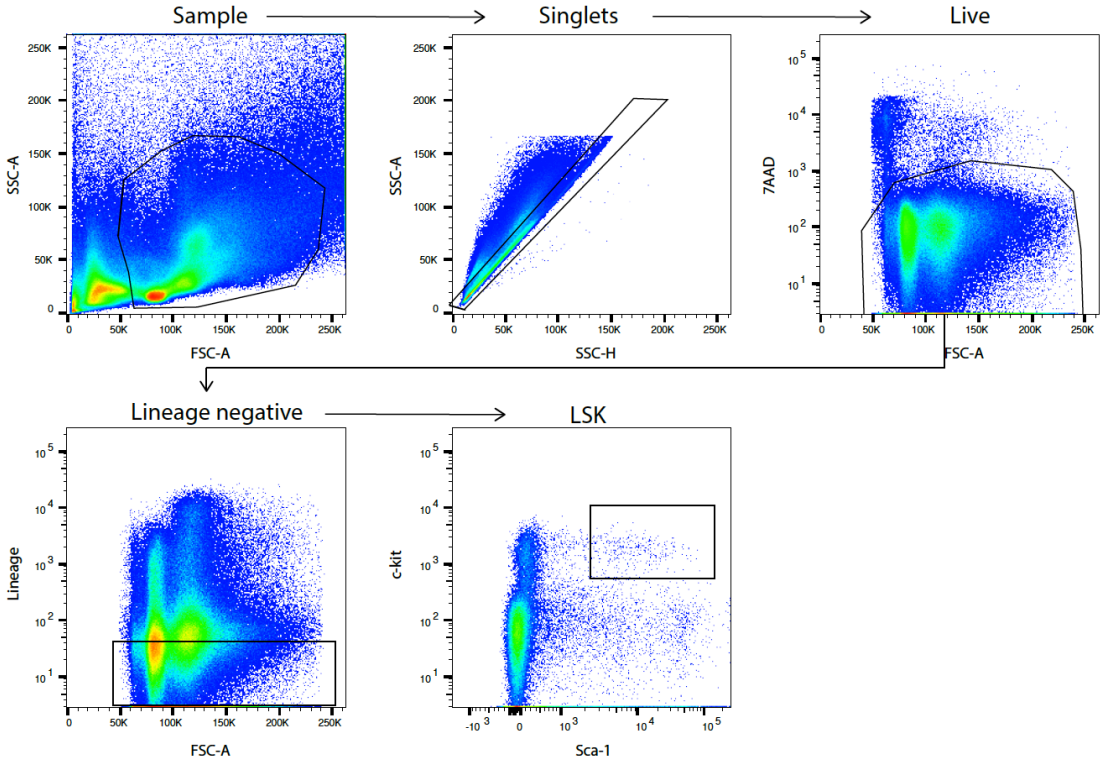


Figure 12. Representative gating strategy for BM and spleen phenotyping of LSK population.
LSK were defined as single live Lineage negative (Lin^-) $Sca\text{-}1^+$ $c\text{-}Kit^+$ cells.

All samples were acquired on LSD Fortessa (BD) and flow cytometry data were analysed by using FlowJo v10 (FLOWJO LLC, Ashland, OR, USA).

2.21 Statistical analysis

Quantitative data in Chapter 4 were analysed using GraphPad Prism 7 software and R. For comparisons between patient and mouse subgroups, unpaired Mann-Whitney test and Kruskall-Wallis were used. Fisher's Exact test was used for proportional MNC subtype analysis of GRO-α flow cytometry data. Kaplan-Meier and Cox proportional hazards modelling (for time-to-event analyses), Random Forest, and Mixed Effects Modelling were done in R (v3.2.2). The R packages were: ggplot2 (v2.2.1), Random Forest (v.4.6-12), rms (vs5.1-0), survival (v2.20-1) and lme4 (v1.1-12). Multivariate analysis included: age at diagnosis, sex, white cell and platelet counts, and (where available and specifically stated in subgroup analysis) mutation status (0/1) for recurrently mutated genes (JAK2, TET2, CALR, MPL, DNMT3A, ASXL1, SRSF2, U2AF1,

IDH2, SF3B1 and CBL), reticulin grade at diagnosis and length of treatment on anagrelide or hydroxycarbamide. These 11 genes all occurred in >2% of patients and have been previously implicated in MPN pathogenesis.

For comparison of cell division kinetics and colony size in Chapter 3 and 5, two-way ANOVA (Sidak's multiple comparison test) was used. For all other p values reported, an unpaired Student's t test was used. For comparison of blood parameters one-way ANOVA (Tukey's multiple comparisons test) was used.

Results

3 The impact of cytokines on haematopoietic stem cell function *in vivo* and *in vitro*

This chapter focused on exploring the impact of extrinsic regulators on individual HSCs with a particular focus on investigating whether or not the presence of specific cytokines in the culture medium is essential for retaining HSC properties *ex vivo*.

Mouse HSCs were cultured *in vitro* under minimal cytokine stimulation for 7 days and were found to be maintained as single cells, prompting us to term the condition a "hibernation" condition. HSC function was tested *in vitro* by assessing cell survival, proliferation kinetics and differentiation potential in liquid culture and colony forming cell (CFC) assays, and *in vivo* by single cell transplantation. To investigate the transcriptional impact of cytokine deprivation and establish the molecular profile of an HSC in the absence of haematopoietic niche signalling, gene expression profiling was performed on hibernating LT-HSCs and compared to freshly isolated counterparts. Additionally, we tested whether genetic modification could be undertaken in hibernation cultures, by transducing individual hibernating HSCs. Finally, the translational potential of this *in vitro* system was assessed by testing its impact on human HSCs.

3.1 HSCs remain in state of hibernation under minimal cytokine stimulation

Different combination of cytokines and growth factors have been used to maintain and expand mouse and human HSCs *in vitro*[95,175,191,194,330–332], with a number of studies suggesting that cytokine signalling is essential for HSC self-renewal and proliferation, but potentially dispensable for stem cell maintenance[135,333,334]. One of the studies supporting this concept has shown that phenotypic HSCs can be maintained as single cells for 5-7 days when lipid-raft clustering is inhibited, resulting in suppressing SCF signalling[135,333]. More recent reports have also demonstrated that minimising the addition of cytokine in culture promote HSC maintenance and quiescence *in vitro* and preserve their function *in vivo*[334].

To investigate more directly the effect of cytokine signalling on HSCs, we tested single HSC function *in vitro* under almost complete absence of exogenous factors. As activation of gp130 signalling by IL-6 or IL-11 has been shown to be essential for effective HSC expansion *in vitro*, IL-11 was the only cytokine added to the minimal culture set up[132,143].

Single mouse BM CD45$^+$EPCR$^+$CD48$^-$CD150$^+$Sca1^{high} (ESLAM Sca1^{high}) long term HSCs (LT-HSCs), ~60% of which can give long-term multilineage reconstitution in single cell transplantation experiments[20] were cultured in the presence of 20ng/mL IL-11 in both serum-containing[257,262] and serum-free conditions[95] (Figure 13).

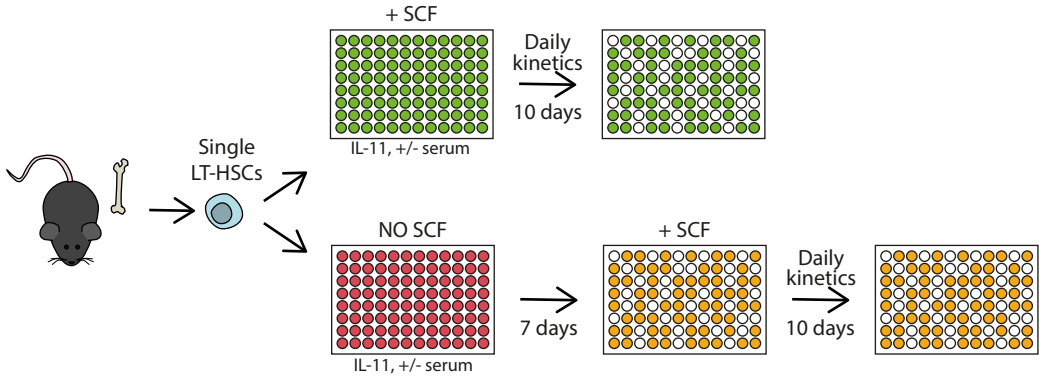


Figure 13. Schematic of single cell *in vitro* culture of mouse HSCs.
Single CD45$^+$EPCR$^+$CD48$^-$CD150$^+$Sca1^{high} LT-HSCs were sorted into individual wells and cultured in the presence of IL-11, in serum-supplemented media or serum-free medium and in the presence of absence of SCF. For SCF-supplemented cultures (green plate), daily cell counts were performed for 10 days. For cultures only containing IL-11 (red plate), HSCs were supplied with SCF on day 7 post-isolation after which daily cell counts were performed for an additional 10 days. In all cases, clone size was assessed at day 10 post-SCF addition.

In both conditions, between 20 and 40% of single LT-HSCs survived 7 days of culture. This demonstrates that the HSC population is heterogeneous in its response to cytokine deprivation and indicates that a proportion of HSCs are more resilient in culture. Unlike HSCs cultured in the presence of TPO or SCF[26,93] where cells divide within the first 2-3 days of division, LT-HSCs cultured in minimal cytokine conditions did not divide during the 7-day culture period with 99.2% (634 of 639 cells) of the surviving input LT-HSCs maintained as single cells for the 7-day culture period (Figure 14).

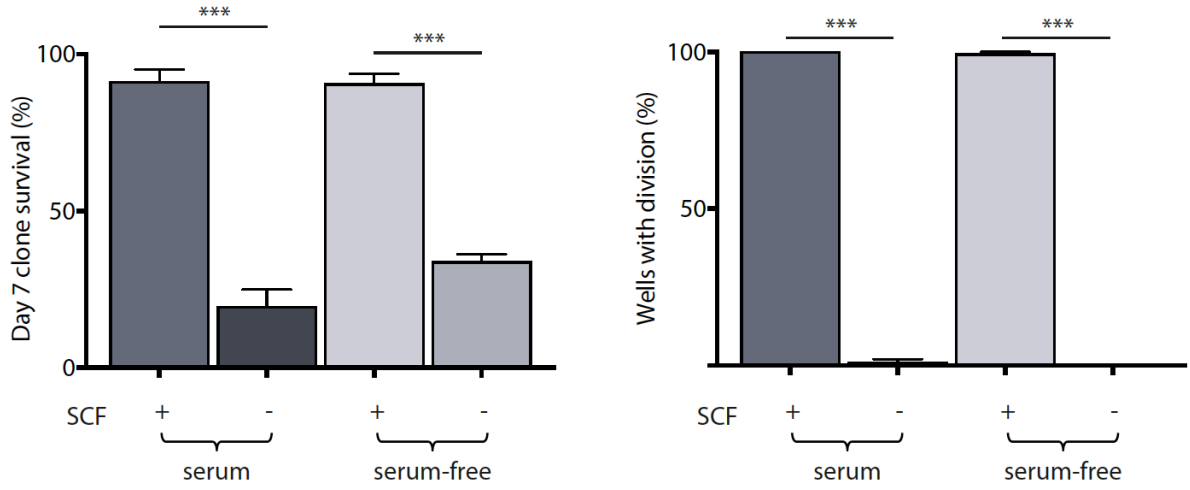


Figure 14. HSCs remain viable single cells in minimal cytokine culture.
Left panel: HSC survival is decreased in the absence of SCF compared to SCF-supplemented medium (+serum/+SCF n=355, 5 biological replicates; +serum/-SCF n=1722, 7 biological replicates; -serum/+SCF, n=144, 2 biological replicates, -serum/-SCF n=284, 3 biological replicates). Right panel: Numbers of wells with >2 cells were scored to determine the number of clones that had divided. At day 7 post-isolation, only culture conditions without SCF retained HSCs as single cells. Bars show mean with SEM. Unpaired t-test: *p<0.05, **p<0.01, ***p<0.001.

This finding was confirmed by time-lapse video monitoring where single cells could be tracked at high temporal resolution allowing confirmation that they did not divide (Figure 15).

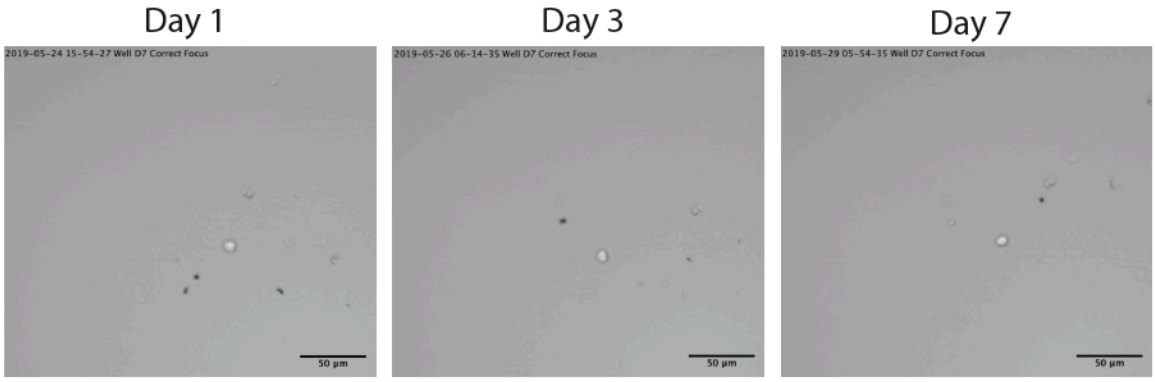


Figure 15. Single-cell time-lapse imaging of single HSCs in hibernation cultures.
Single $CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca1^{high}$ (ESLAM $Sca1^{high}$) LT-HSCs were sorted into individual wells of a 96-well plate and cultured in the presence of IL-11, in serum-supplemented media, in the absence of SCF. Each well was imaged every 50 minutes for 7 days. The three pictures show an individual well imaged on day 1, 3 and 7 of culture in the hibernation condition. The single cell did not divide over the course of the 7-day hibernation culture. Scale bar represents 50µm.

Together, these data prompted us to define the minimal cytokine condition as a "hibernation" condition, similar to the cellular state of LT-HSCs described previously following the addition of lipid raft inhibitors to inhibit cytokine signalling[135]. From here on, the 7-day surviving input LT-HSCs will be referred to as "hibernating HSCs" (hibHSC).

3.2 Minimal cytokine culture conditions maintain HSC heterogeneity in cell cycle kinetics

The vast majority of HSCs reside in the G_0 phase of the cell cycle when isolated from the BM. Cytokine stimulation *in vitro* subsequently pushes them out of their quiescent state with a first division typically occurring within the first 24-48 hours[335,336]. To assess the functional potential of day 7 single hibernating LT-HSCs, 300ng/mL SCF was added to each well, in order to stimulate HSCs to divide, and to mirror the cytokine combination previously studied in freshly isolated LT-HSCs[257,262] (Figure 13). Time to first and second division of hibernating HSCs post-stimulation overlapped nearly identically with freshly isolated LT-HSCs receiving SCF straight after isolation (Figure 16, left panel). Moreover, clonal proliferation over the subsequent 10 days was highly similar to freshly isolated HSCs stimulated for 10 days, as measured by assessing clone size distribution (Figure 16, right panel). These data demonstrated that hibernating HSCs maintain heterogeneity in cell cycle after SCF addition and that minimal cytokine culture condition do not select HSCs based on cell cycle state.

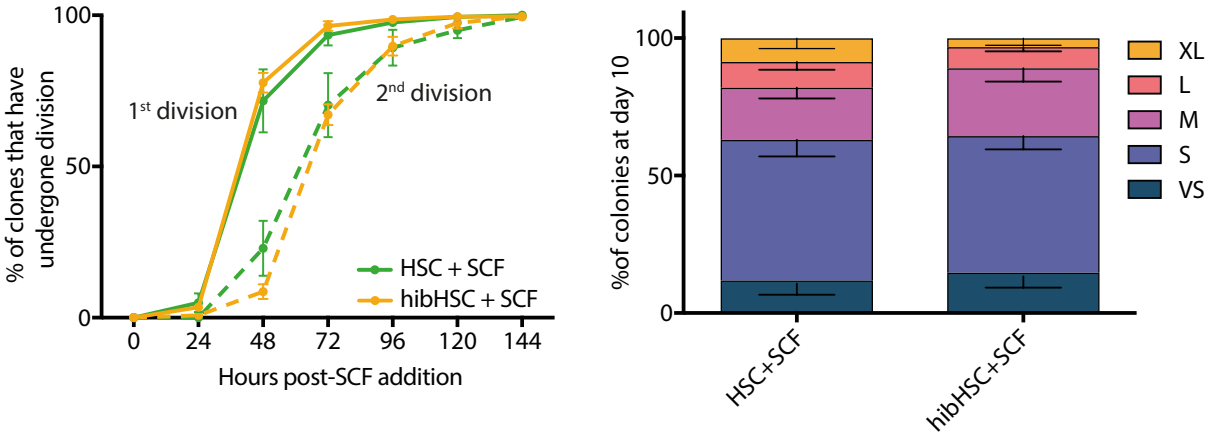


Figure 16. Hibernating HSCs (hibHSC) retain heterogeneity in cell division kinetics and clone size in hibernation conditions.
Left panel: entry into cell cycle was comparable between freshly isolated HSCs (HSC, green solid line) and cells that had been maintained as single cells for 7 days (hibHSC, orange solid line). Time to subsequent cell division (dotted lines) was not significantly different between conditions (Stem Cell Factor (SCF) added at day 0, n=355, 5 biological replicates; SCF added at day 7, n=1722, 7 biological replicates). Two-way ANOVA (Sidak's multiple comparison test). Right panel: colony size was measured on day 10 post-SCF addition and no differences in clone size distribution was observed between HSCs cultured in presence of SCF from day 0 and post-hibernation HSCs (7 days +10 days post-SCF addition in culture). Two-way ANOVA (Sidak's multiple comparison test): VS=very small, p>0.9999; S=small, p=0.9998; M=medium, p=0.6736; L=large, p>0.9990; XL=very large, p>0.9999. All bars show mean with SEM.

To assess whether hibernating HSCs also maintain their multi-lineage potential, 7-day single cells were transferred into CFC assays for a further 14 days before being counted, typed and subsequently harvested for confirmatory flow cytometry analysis (Figure 17).

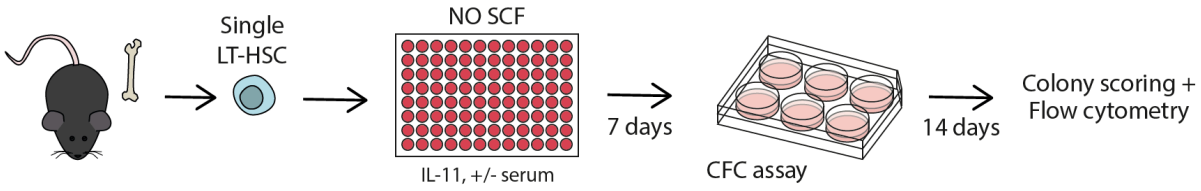


Figure 17. Schematic of CFC assay on single hibernating HSCs.
Single CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca1^{high} (ESLAM Sca1^{high}) LT-HSCs were cultured for 7 days in IL-11 alone, in serum-supplemented or serum-free medium. After 7 days, single hibernating LT-HSCs were individually transferred into a cytokine rich methyl-cellulose CFC assay and cultured for an additional 14 days. On day 14, colonies were manually scored, and lineage composition of individual colonies was also assessed by flow cytometry.

Overall, 60-70% of single hibHSCs in serum free or serum-containing conditions were able to generate clones with the majority generating at least three different mature cell types as determined by flow cytometry for cell surface markers Ly6g (Gr1), CD11b (Mac-1), Ter-119 and CD41 (Figure 18).

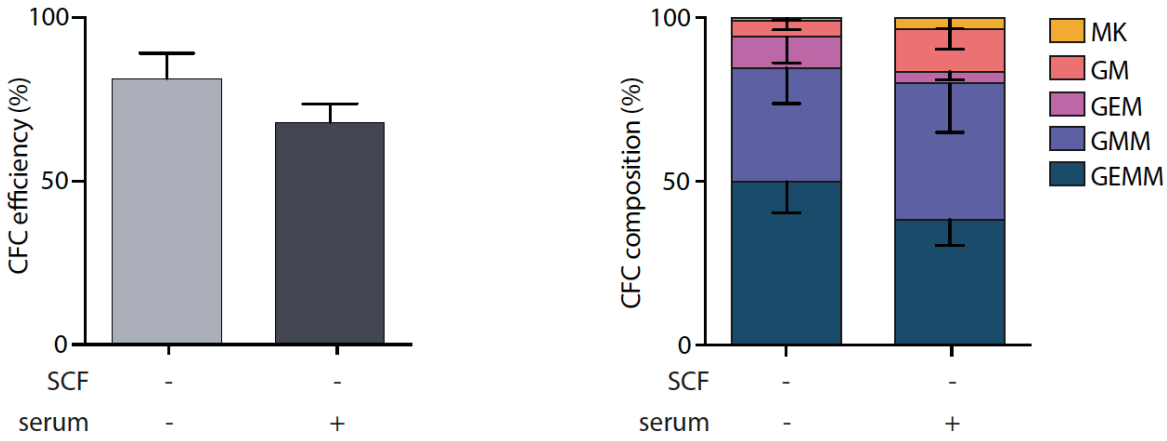


Figure 18. hibHSCs retain multi-potency *in vitro*.
Left panel: CFC assay efficiency for single LT-HSCs in serum-supplemented and serum-free hibernating cultures (serum-free n=121, 5 biological replicates; +serum n=230, 6 biological replicates). Right panel: Colony subtype analysis showed that the majority of single cells (~80%) generated colonies of at least three lineages in CFC assays (serum free n=70, 4 biological replicates; +serum n=166, 3 biological replicates). Colonies were defined as MK (containing cells positive for megakaryocyte marker CD41), GM (containing cells positive for granulocyte/monocyte markers Gr1 and Cd11b), GEM (positive for GM and erythrocyte markers Gr1, Cd11b, and Ter-119), GMM (positive for GM and MK markers), and GEMM (positive for GM, MK and E markers), as described in Methods, section 2.5. Bars show mean with SEM.

These results demonstrate that LT-HSCs can survive in minimal cytokine conditions without undergoing cell division and they can be revived to normal function by re-introducing a mitogen (e.g., SCF) into the culture system. Importantly, after a period of induced hibernation, the *in vitro* properties of these "resilient" HSCs are preserved, making them indistinguishable from the freshly isolated HSCs.

3.3 Hibernating single HSCs retain complete *in vivo* repopulation capacity

Although *in vitro* colony assays permitted the evaluation of which mature cell lineages the colonies contained, CFC assays do not measure the cell's capacity to differentiate into lymphoid lineages and are not capable of assessing self-renewal[337]. In order to assess whether hibernation conditions impacted HSC self-renewal expansion

capacity, single day-7 hibHSCs (from both serum-supplemented and serum-free conditions) were transplanted into sub-lethally irradiated mice and their repopulation capacity was compared to freshly isolated HSCs as outlined in Figure 19.

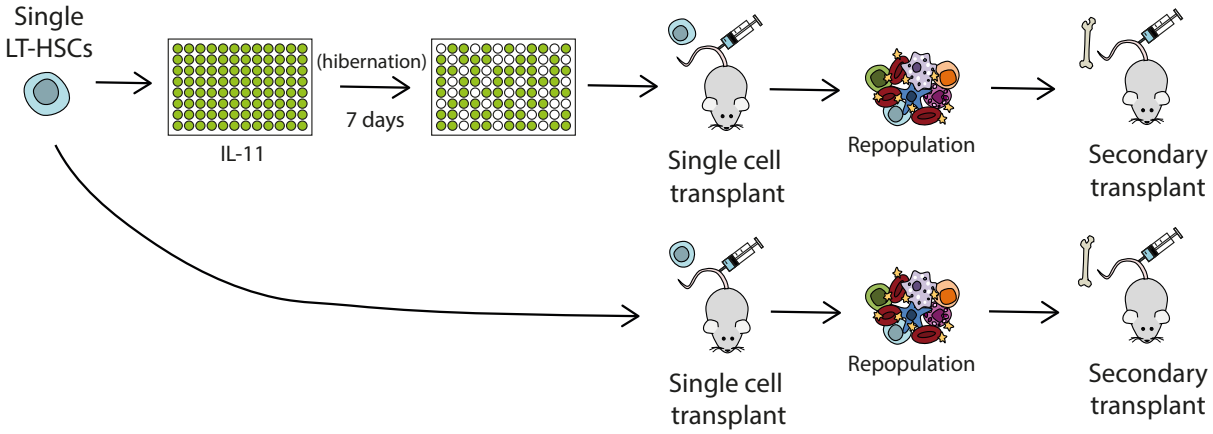


Figure 19. Schematic of single-cell transplantation of fresh and hibHSCs.
Single $CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca1^{high}$ (ESLAM $Sca1^{high}$) LT-HSCs were cultured in hibernation conditions in either serum-supplemented or serum-free medium. Single fresh HSCs or day 7 hibHSCs were transplanted into W41-CD45.1 recipients (fresh n=69, serum-free n=24, +serum n=29). Secondary transplantations were undertaken in all mice with donor engraftment (>1%) at 16-24 weeks post-transplantation.

The single cell transplantation assays revealed that 62.5% (15/24) and 45.8% (13/29) of primary recipients transplanted with single hibHSCs (without serum and with serum respectively) could give rise to greater than 1% multi-lineage donor chimaerism at 16-24 weeks post-transplantation, compared to 48.8% (33/69) of freshly isolated HSCs (Figure 20). 69.23% of the single freshly isolated HSCs which repopulated primary recipients, were able to repopulate secondary recipients, compared to 93.3% of the hibHSC(+serum) and 78.57% of hibHSC(serum-free) (Figure 20), suggesting that the 7-day hibernation period *in vitro* did not negatively impact HSC self-renewal.

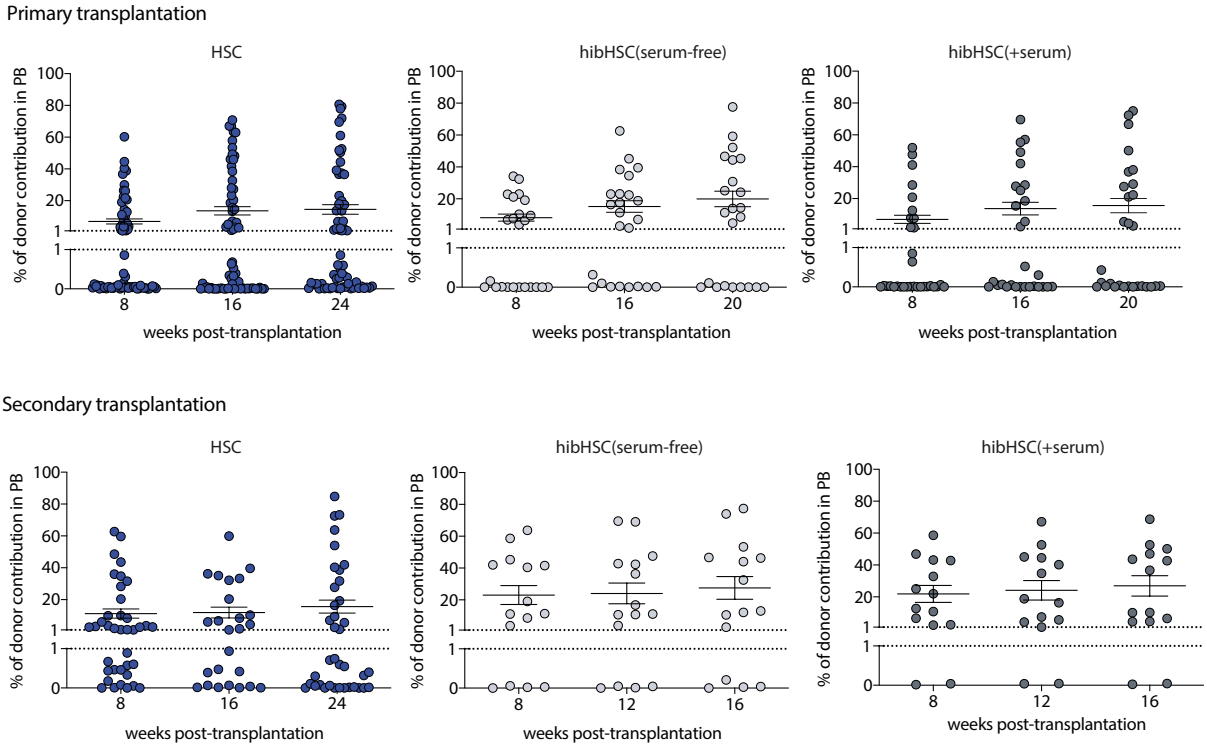


Figure 20. *In vivo* HSC functional activity of single hibHSCs is preserved in both primary and secondary recipients.
Percentage of donor chimerism in the peripheral blood of primary recipient mice at 8, 16, and 24-week post-transplantation, and secondary recipient mice at 8-, 12/16- and 24-weeks post-transplantation. For the secondary transplantation, values in the graph are shown as a mean of the 2-3 mice transplanted with BM cells from the same primary donor. Recipients with chimerism >1% and at least 0.5% of GM, B, and T cells were considered repopulated. Among the primary "HSC" transplanted mice, 5 recipients died before week 24, among the "hibHSC(+serum)" transplanted mice, 3 recipients died before week 12, all for non-experimental reasons. Among the secondary "HSC" transplanted mice, 11 mice died before week 24 for non-experimental reasons, 23 mice were not bled at week 16, however these mice were repopulated at week 8 and were all still positive at week 24. Among the secondary hibHSC(+serum) recipient mice, 1 mouse died before week 16 post-transplantation, for non-experimental reasons.

3.4 Absence of cytokines in hibernation conditions does not alter lineage output in single-cell transplantation

A method to classify HSCs depending on the relative contribution of individual HSC outputs to the total circulating cell numbers was introduced by Dykstra et al.[46] (see section 1.1.3.1). HSCs were grouped into "lymphoid-deficient" (α), "balanced" (β), or "myeloid-deficient" (γ and δ) subtypes, based on their relative production of mature myeloid and lymphoid cells in the post-transplantation period. While α and β HSCs possess robust multi-lineage reconstitution in both primary and secondary

recipients, γ/δ fail to repopulate secondary recipients and typically have reduced levels of donor cells in the latter stages of primary transplantations.

Considering this large difference in the quality of HSCs, we were interested in asking whether the hibernation conditions selectively retained or preserved particular HSC subtypes, especially considering that previous culture conditions have been shown to be largely skewed toward producing HSCs with less functional potential (i.e., γ/δ HSCs)[46]. In order to investigate whether differences in HSC subtypes were observed between hibernating and freshly isolated HSCs, we classified HSC subtypes produced in single cell transplantation experiments (Figure 21). No significant difference in the proportion of HSC subtype was observed between freshly isolated and cultured HSCs, indicating that the 7-day hibernation period did not lose the HSCs with durable self-renewal activity, as also supported by the secondary transplantation data displayed above (Figure 20). Interestingly, there was a slight skewing towards α-HSCs in terms of proportion, but additional transplantations would need to be undertaken to further assess this possibility.

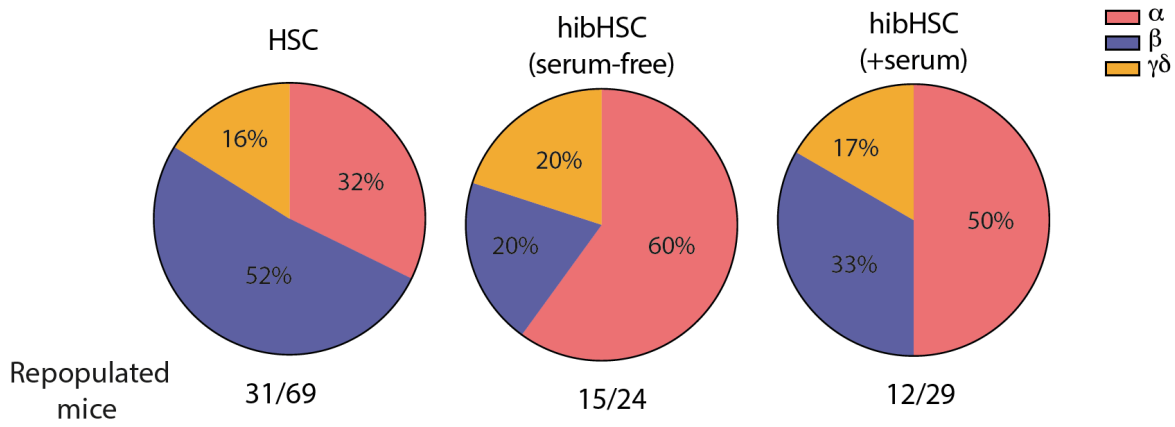


Figure 21. Distribution of HSC subtypes in mice transplanted with HSCs and hibHSCs. The founder HSCs was retrospectively assigned one of the following subtypes: α (alpha, M/L>2), β (beta, 0.25>M/L<2), γ (gamma, M/L<0.25), δ (delta, M/L<0.25 and failure to contribute to myeloid lineage post 16 weeks) in accordance with Dykstra et al., 2007[46] (HSC n=31/69; hibHSC(serum-free) n=15/24; hibHSC(+serum) n=12/29). No significant difference was observed in the balance of mature cell outputs between HSCs and hibHSCs(serum-free) or HSCs and hibHSCs(+serum), based on donor myeloid (M) to lymphoid (L) ratio at 16 weeks post-transplantation in primary recipients. Two-way ANOVA (Tukey's multiple comparison test).

Together, these results show that in the absence of a haematopoietic niche, and the nearly complete removal of supportive cytokines, LT-HSCs cultured under hibernation conditions fully preserve their functional output both *in vitro* and *in vivo.*

3.5 Hibernating LT-HSCs can be transduced without undergoing division

The knowledge that LT-HSCs are fully functional during the hibernation cultures offers a potential window *in vitro* with valuable experimental and clinical potential, where hibernating HSCs could be manipulated, and the impact of specific modifications assessed. In order to explore this possibility, we assessed whether transgenes could be delivered during the hibernation period. Small bulk populations of LT-HSCs were isolated from mouse BM and transduced with a GFP-containing lentivirus. Cells were cultured for 2 days in hibernation conditions and then re-sorted into single cell cultures, in the presence of SCF, to determine single cell transduction efficiencies and survival (Figure 22).

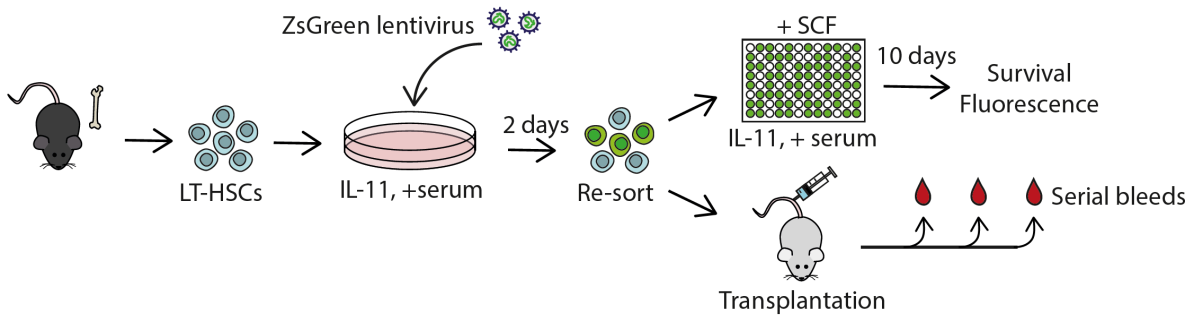


Figure 22. Schematic of lentiviral transduction of hibernating HSCs.
CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca-1^{high} (ESLAM Sca1^{high}) LT-HSCs were isolated and transduced with ZsGreen lentivirus and cultured together for 2 days in StemSpan supplemented with 10% serum and 20ng/mL IL-11. Cells were collected and virus was removed by collecting and re-sorting the cells into single wells and cultured in SCF-supplemented (300ng/mL) media for an additional 10 days. Transduction efficiency was assessed by visual inspection on a fluorescent microscope. 4001 total viable cells (a mixture of transduced and non-transduced cells) were resorted and transplanted into W41-CD45.1 mice (n=6 recipients) and donor contribution and GFP expression were assessed by serial bleeds and flow cytometry analysis.

After 10 days, 40% of the original sorted cells (284/657) successfully produced colonies and ~17.6% (50/284) of the surviving clones were GFP^{+} (Figure 23, left panel). To assess whether the virus was delivered to LT-HSCs that retained their functional properties, cells were harvested following the 2-day transduction and transplanted to be assessed for GFP^{+} donor cell repopulation at 4-, 8-, and 16-weeks

post-transplantation. All recipient mice were positive with initial reconstitution levels ranging from 2 to 6% GFP+ cells and this contribution was stable throughout the monitoring period (Figure 23, middle and right panel). Together, these data formally demonstrated that lentiviral constructs can be successfully delivered to hibernated HSCs which have not undergone cell division. However, *in vitro* expression of GFP vector requires SCF stimulation, and therefore cell division.

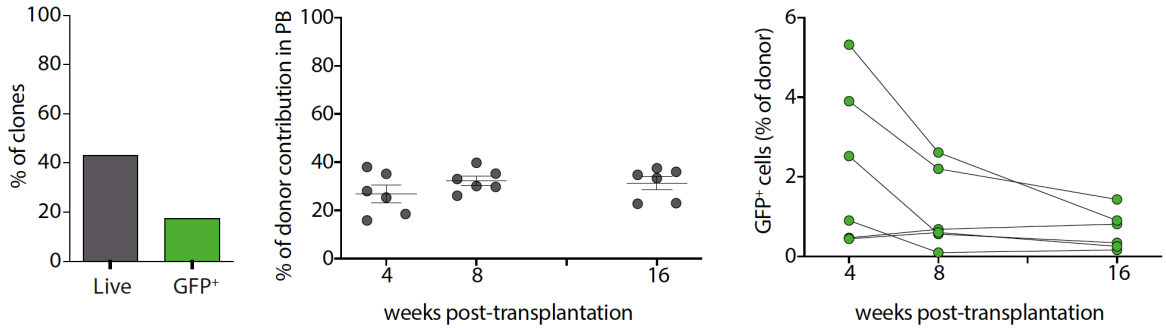


Figure 23. Single hibHSCs can be manipulated by lentiviral transduction.
Left panel: grey bar shows the percentage of clone surviving after 10 days post-addition of SCF, and the green bar indicates the percentage of GFP+ clones (n=672, 1 biological replicate). Middle and right panel: chimerism levels (60-70%) were stable across all recipients at all time points, and 1-2% of donor cells were positive for GFP at 16-week post-transplantation.

3.6 Gene expression analysis reveals a core gene expression programme shared between hibHSCs and freshly isolated HSCs

In vitro and *in vivo* data have demonstrated clearly that LT-HSCs deprived of SCF in hibernation conditions retain their functional properties, including the ability to reconstitute primary and secondary recipients at the same frequency as freshly isolated LT-HSCs. Aside from IL-11 stimulation, these LT-HSCs were cultured in the complete absence of signals from the haematopoietic niche or any neighbouring cells. Therefore, the transcriptome of these cells could be a useful comparator for determining which genes might be dispensable for LT-HSC function, assuming that those genes shared between freshly isolated HSCs and hibHSCs are those essential for HSC function. Moreover, comparing the gene expression programmes of hibHSCs upon activation might lend further insight into how signalling pathways are differently activated/deactivated following a period of niche independence.

To address these questions, we performed single-cell gene RNA-sequencing on LT-HSCs cultured in hibernation conditions for 7 days (hibHSC, n=106) and compared them to freshly isolated single LT-HSCs (HSC, n=165) and also to HSCs and hibHSCs stimulated with SCF for 16 hours (HSC+SCF(n=63), hibHSC+SCF(n=127)) to determine the common pathways of activation upon SCF stimulation.

The broad gene expression patterns of single HSCs from the 4 different conditions were first compared using Uniform Manifold Approximation and Projection (UMAP) in order to visualise molecular clusters and identify distinct gene expression signatures between cell populations. Cells from each physiological setting clustered together in a unique space, indicating that while there is substantial similarity to the molecular profile of freshly isolated HSCs, there are some molecular changes which result from HSCs being removed from the *in vivo* microenvironment for 7 days (Figure 24).

Still, our functional data demonstrated that the hibHSCs fully retain their HSC properties, offering a powerful comparator to identify genes essential for HSC function.

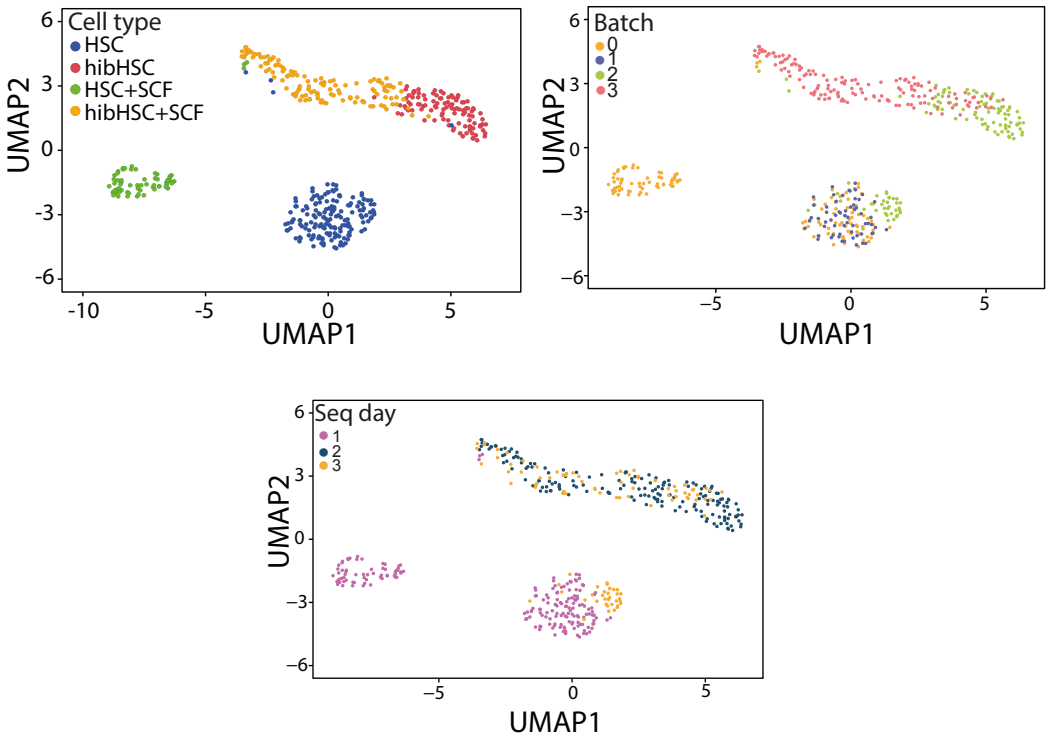


Figure 24. UMAP of single-cell gene expression profiling shows distinct molecular regions of freshly isolated HSCs and hibHSCs.
Uniform Manifold Approximation and Projections (UMAP) depicting (upper left) cell type (freshly isolated HSCs (HSC), blue dots; hibernating HSCs (hibHSC), red dots; freshly isolated HSCs stimulated with Stem Cell Factor(SCF) for 16 hours (HSC+SCF), green dots; hibernating HSCs stimulated with SCF for 16 hours (hibHSC+SCF), orange dots, (upper right) batches (batch 0, orange dots; batch 1, blue dots; batch 2, green dots; batch 3, pink dots); (bottom) days batches were sequenced (day 1, purple dots; day 2, blue dots; day 3, orange dots).

In their 2015 paper, Wilson et al. identified a gene signature shared between single phenotypic HSCs isolated using five different HSC sorting strategies[27,313,338,339]. Combining the individual gene expression profiles with the expected proportion of functional HSCs in each fraction, they were able to determine a set of putative HSCs that expressed a set of specific genes and termed the signature "MolO" (molecular overlap signature). The MolO gene signature includes 28 genes that are differentially expressed in the MolO population, compared to the cells that did not molecularly overlap (NoMO), and this signature comprises transcription factors involved in self-renewal and differentiation, epigenetic regulators, as well as cell surface markers.

To further explore the similarity of hibHSCs to freshly isolated HSCs, we compared the expression levels of key HSC regulators that comprise the MolO gene signature.

MolO scores are indicative of the confidence level of determining that an individual cell is in fact a MolO cell. Overlaying MolO scores on the UMAP plot shows that the highest MolO scores are present in the freshly isolated HSCs and in the hibHSCs, followed by their SCF-stimulated counterparts (Figure 25, left graph). This pattern is mirrored in the violin plots displaying individual single cell MolO scores by physiological conditions (Figure 25, right graph).

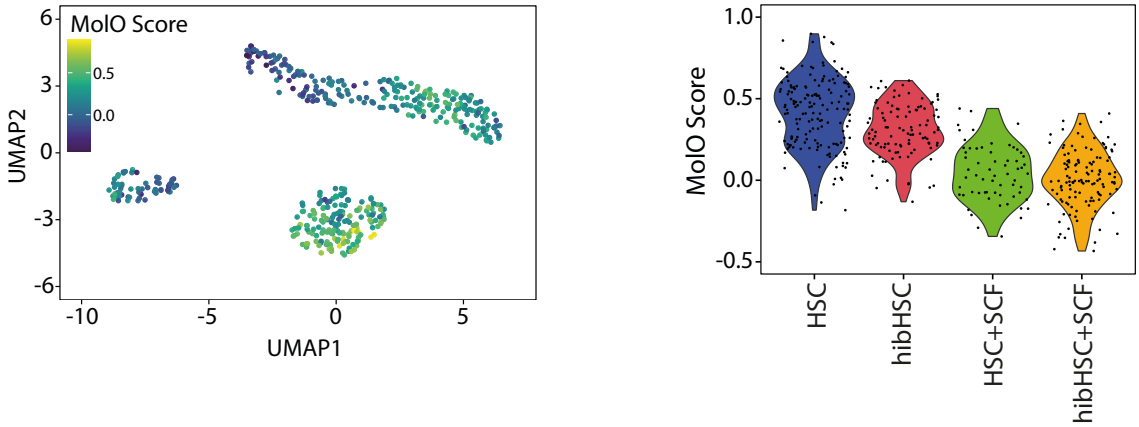


Figure 25. MolO gene signature in HSC and hibHSC populations.
Left panel: the HSC-specific molecular overlap (MolO) gene signature score was computed based on average expression of signature genes and projected onto the Uniform Manifold Approximation and Projections (UMAP) distribution. Right panel: violin plots showing MolO scores for individual HSCs in each physiological state with the HSCs and hibHSCs having the highest overall scores. HSC=freshly isolated HSCs, hibHSC=hibernating HSCs, HSC+SCF=freshly isolated HSCs stimulated with SCF for 16 hours, hibHSCs+SCF=hibernating HSCs stimulated with SCF for 16h post-hibernation.

The relatively high MolO scores in hibHSCs indicate the utility of the MolO score for identifying functional HSCs irrespective of their physiological state. That said, the similarity in these molecular features also suggests that other factors must be contributing to the molecular separation between freshly isolated HSCs and hibHSCs. The relative expression of all the MolO genes across the four different conditions is shown in Figure 26.

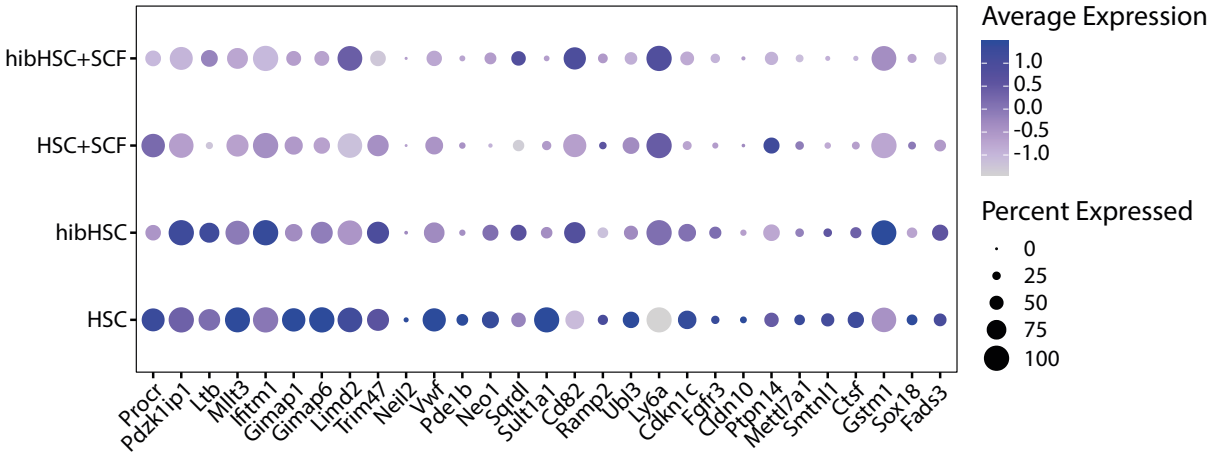


Figure 26. MolO gene relative expression in HSC, HSC+SCF, hibHSC, hibHSC+SCF.
Dot plot representing the average normalised expression of MolO genes in HSCs (HSC), hibernating HSCs (hibHSC), freshly isolated HSCs stimulated with SCF for 16 hours (HSC+SCF), and hibernating HSCs stimulated with SCF for 16h post-hibernation (hibHSCs+SCF). The size of each dot indicates the proportion of cells with normalised expression level >0 (scaled expression represented by colour intensity).

Similarity between hibHSCs and freshly isolated HSCs was also evident when components of the cell cycle machinery were assessed to predict the cell cycle stage of each profiled LT-HSC[51,340]. Displaying cell cycle state on the UMAP clustering shows that both freshly isolated and hibHSCs reside in the G_0/G_1 phase of the cell cycle and demonstrate again that cell cycle status is not the primary driver of molecular differences between the two populations. More than 80% of freshly isolated HSCs and hibHSCs had molecular profiles consistent with being in the G_0/G_1 phase of the cell cycle, whereas both SCF-stimulated HSC fractions had fewer than 40% G_0/G_1 cells (Figure 27). These results are in line with the cell cycle kinetics observed in Figure 16, where cells that divide early in the curve (i.e., between 20 and 30 hours post-stimulation) would be expected to have progressed to the S or G2 phase by 16 hours post-stimulation.

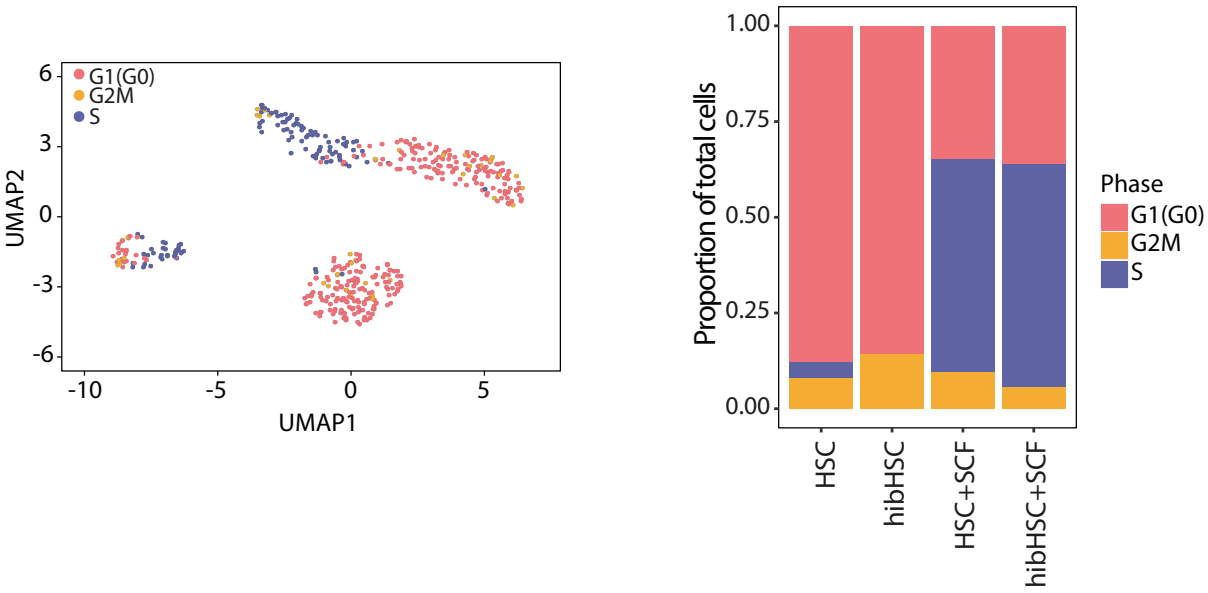


Figure 27. Cell cycle stage of individual HSCs.
Left panel: cell cycle scores were computed for each cell and identified states were projected on the Uniform Manifold Approximation and Projections (UMAP) distribution (G1(G0), pink; G2/M, orange; S, blue). Right panel: proportional representation of cell cycle stages of all cells within each distinct population (G1(G0), pink; G2/M, orange; S, blue). HSC=freshly isolated HSCs, hibHSCs=hibernating HSCs, HSC+SCF=freshly isolated HSCs stimulated with SCF for 16 hours, hibHSC+SCF=hibernating HSCs stimulated with SCF for 16 hours.

3.7 Resolving the list of functional HSC regulators

Despite the strong overlap in cell cycle and MolO gene signature expression, hibHSCs form a distinct cluster away from freshly isolated HSCs (Figure 24). To further explore the origin of this divergence, we performed global differential gene expression analysis between HSCs and hibHSCs and identified 116 upregulated and 138 downregulated genes. The top 25 genes upregulated or downregulated in HSCs compared to hibHSCs are shown in Table 4 and **Error! Reference source not found.** respectively.

Table 3. List of the top 25 genes upregulated in hibernating HSCs vs freshly isolated HSCs.

Term	p-value	Adjusted p-value	Average logFC
Wfdc17	9.10E-177	1.68E-172	7.10322235
Dlk1	2.08E-42	3.84E-38	4.49895846
Plscr2	4.23E-20	7.80E-16	4.32551081
Serpinb1a	4.08E-29	7.53E-25	4.28012118
Slc7a11	5.49E-35	1.01E-30	4.03899542
Rufy4	3.50E-37	6.45E-33	4.03784027
Abca4	5.05E-45	9.31E-41	4.03171896
Rtl1	2.59E-14	4.77E-10	3.76905583
Hbb-bt	3.41E-21	6.29E-17	3.63174278
Vldlr	9.98E-14	1.84E-09	3.62450248
Nqo1	1.29E-20	2.38E-16	3.56907444
Muc1	1.03E-10	1.90E-06	3.54745799
Ahnak2	3.30E-33	6.09E-29	3.43071039
Ier3	2.93E-26	5.41E-22	3.42514716
Socs3	2.93E-21	5.40E-17	3.42490512
Bex6	1.66E-09	3.06E-05	3.38460344
Lox	4.01E-21	7.41E-17	3.19900896
Gdf15	4.83E-11	8.91E-07	3.11421388
Hbb-bs	1.82E-17	3.36E-13	3.11316968
Pdcd1lg2	2.99E-11	5.51E-07	3.08694434
Il1r1	3.75E-09	6.91E-05	3.03718687
Klrb1c	6.88E-08	0.00126872	2.92472786
1700025G04Rik	9.45E-10	1.74E-05	2.88216205
Selp	9.13E-11	1.68E-06	2.87643085
S100a4	1.95E-18	3.60E-14	2.77000567

Table 4. List of the top 25 genes downregulated in hibernating HSCs vs freshly isolated HSCs.

Term	p-value	Adjusted p-value	Average logFC
Slc34a2	5.45E-190	1.01E-185	-7.1376404
Fos	5.21E-32	9.61E-28	-4.7520421
H2-Aa	6.02E-27	1.11E-22	-4.1974801
Ccnd1	4.09E-50	7.54E-46	-3.9396108
Egr1	2.37E-30	4.37E-26	-3.3892439
Vegfc	7.33E-27	1.35E-22	-3.347791
Slc13a2	1.36E-14	2.52E-10	-3.1745353
Rfng	7.82E-09	0.00014428	-3.0819143
Scn4b	1.00E-68	1.85E-64	-2.9911376
Trib2	7.30E-15	1.35E-10	-2.9158093
Etv5	3.08E-21	5.68E-17	-2.9145957
Dusp6	1.23E-21	2.27E-17	-2.8594844
Cx3cl1	1.07E-10	1.97E-06	-2.8244415
D630039A03Rik	3.79E-16	7.00E-12	-2.7530488
Hdac9	4.63E-14	8.54E-10	-2.7361983
Rarb	6.51E-16	1.20E-11	-2.7253267
Ydjc	1.07E-13	1.98E-09	-2.7121813
Ablim1	1.06E-24	1.96E-20	-2.7071264
H2-Eb1	5.22E-11	9.63E-07	-2.6595083
Zfp2	9.13E-11	1.68E-06	-2.6422978
Abcg3	1.86E-21	3.42E-17	-2.6228241
Plk2	5.21E-14	9.62E-10	-2.5669092
Rpl13-ps3	1.14E-77	2.10E-73	-2.5514761
Gbp4	1.00E-12	1.85E-08	-2.5075769
Gbp9	2.26E-16	4.17E-12	-2.484421

Amongst those additional genes whose expression was significantly reduced, a number of AP-1 complex members were identified, including *Jun* and *Fos* and their co-regulator *Ncor2*, stress-response and apoptosis factors, which have been recently suggested as potential regulator of HSC self-renewal, via the JNK pathway[341]. Two other important examples are *Cish*[342] and *Vwf*[61], which have been previously described in HSC biology (Figure 28).

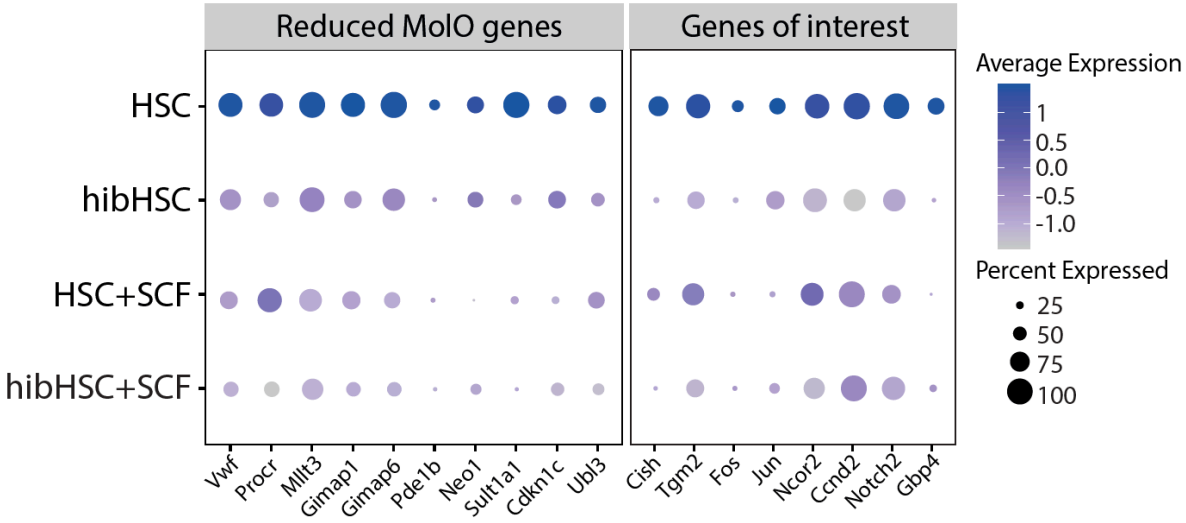


Figure 28. Genes downregulated in hibHSCs vs HSCs.
Dot plot representing the average normalised expression of a selection of MolO genes and genes of interest which are downregulated in hibernating HSCs (hibHSC) compared to freshly isolated HSCs (HSC). The size of each dot indicates the proportion of cells with normalised expression level >0 (scaled expression represented by colour intensity). HSC=freshly isolated HSCs, hibHSC=hibernating HSCs, HSC+SCF=freshly isolated HSCs stimulated with SCF for 16 hours, hibHSC+SCF=hibernating HSCs stimulated with SCF for 16 hours.

On the other hand, consistent with being kept in minimal cytokines, a number of stress response and nutrient deprivation pathways were upregulated in hibHSCs, including cAMP[343]and mTOR signalling, alongside Glycolysis and Fatty Acid Biosynthesis, as shown by KEGG pathway analysis (Figure 29).

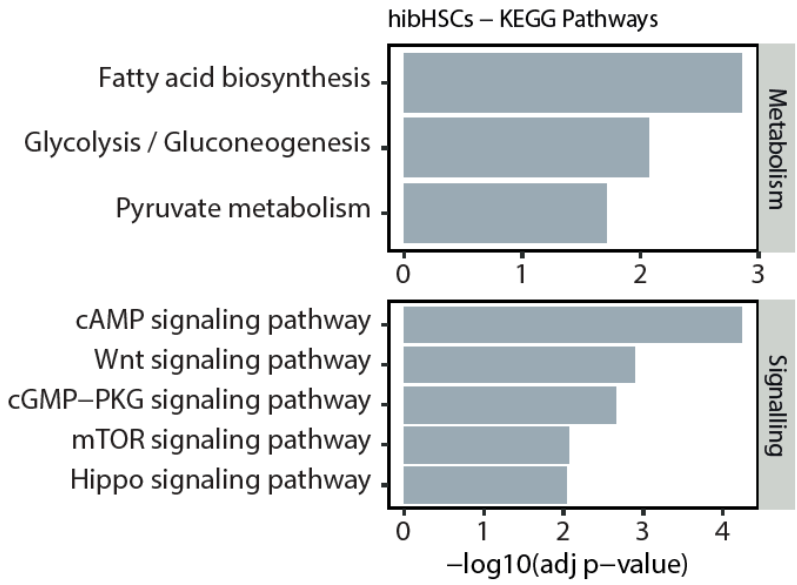


Figure 29. Upregulated pathways in hibHSCs vs HSCs.
KEGG pathway enrichment in unstimulated hibernating HSCs (hibHSC), showing selected metabolic and signal transduction pathways (enrichment cut-off: adjusted p-value >0.05).

Interestingly, pro-survival genes *Ier3* and *Pdcd1lg2* were expressed in hibernating HSCs, alongside multiple HLF target genes, including *Lyz1* and *Lrcc8a*, potentially supporting the idea that hibHSCs are exerting a stress response to survive[344] and preserve their function, in response to cytokine deprivation (Figure 30).

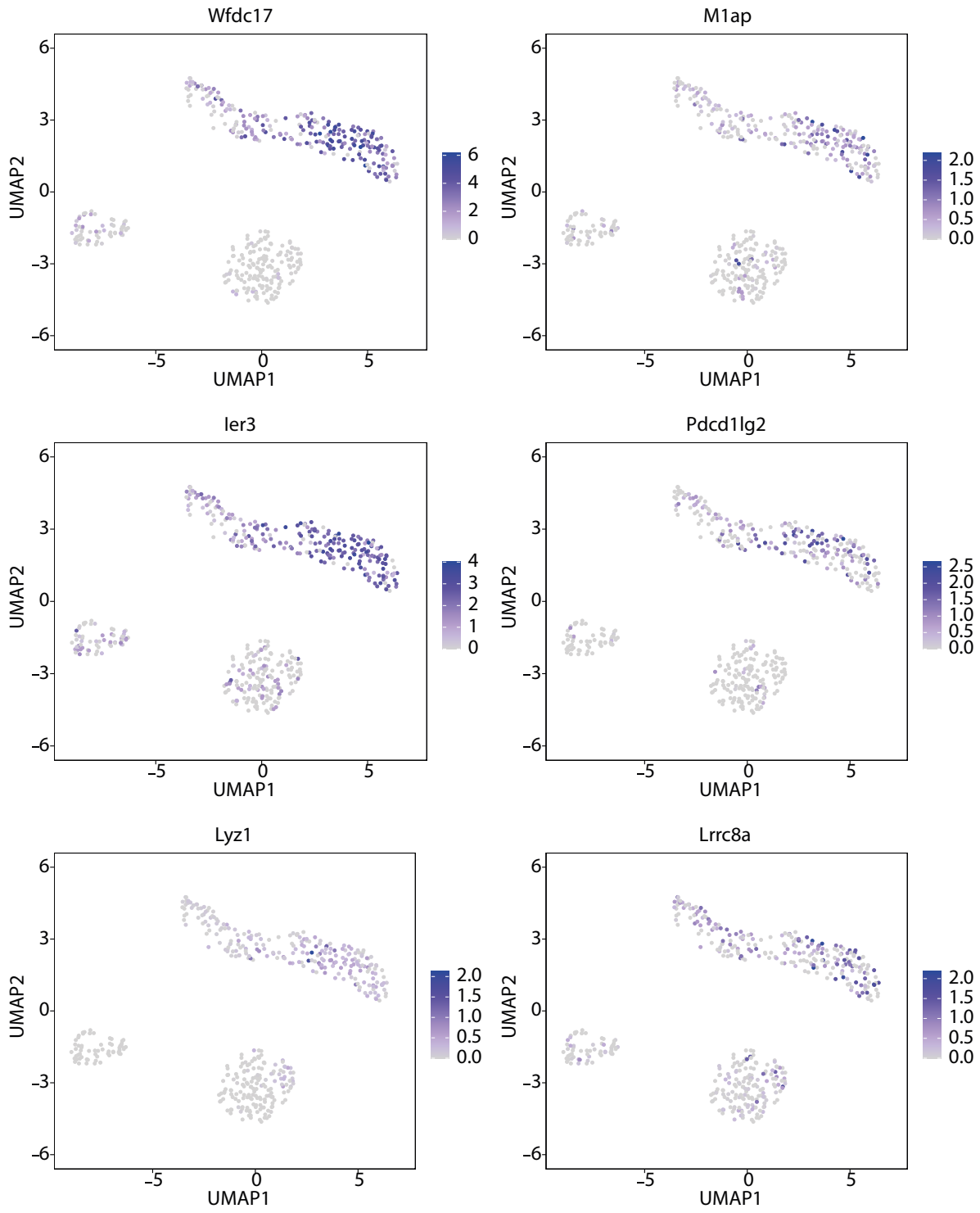


Figure 30. Genes of interests enriched in hibHSC.
Uniform Manifold Approximation and Projections (UMAPs) of selected genes of interests which are upregulated in hibHSCs (manually selected from differential expressed (DE) gene set). The large majority of the hibHSCs appear in the upper right portion of the plot (see Figure 24 for reference).

Altogether these results enforce the idea that despite the high functional overlap between HSCs and hibHSCs, a number of molecular differences exist between these two populations. Importantly, since hibHSCs retain their functional properties *in vivo*, the downregulation of a number of self-renewal regulators in hibHSCs might indicate that such factors are not essential for HSC *in vitro* and *in vivo* function.

3.8 Hibernation cultures resolve common targets of SCF activation

Historically, the molecular impact of individual cytokines on HSCs has been investigated following their isolation from their *in vivo* environment. However, the impact that membrane dynamics, protein turnover, and transcriptional priming would have on an HSC in response to a particular exogenous signal remains unclear. Hibernation cultures offer the possibility to study a different physiological state of HSCs from which to understand the direct impact of adding exogenous cytokines to functional HSCs. Using SCF as a stimulus, we profiled freshly isolated HSCs and hibHSCs to identify individual gene expression patterns associated with cytokine stimulation (HSC+SCF, hibHSC+SCF). First, we generated a list of differentially expressed genes from the HSC vs HSC+SCF and hibHSC vs hibHSC+SCF comparison. 27 genes were commonly differentially expressed (13 up-regulated and 14 down-regulated) upon addition of SCF (Figure 31) An expected activation of ATP generation and nucleotide metabolism is observed, alongside a number of positive cell cycle mediators (*Mcm2, Mcm4, Mcm10, Rad51, Rad51ap1*) and a reduction in developmental and MAPK-mediated signalling. In addition to these expected changes, this system also allows us to identify SCF targets specifically induced in HSCs and show that expression of *Mif*[345] (an inflammatory cytokine promoting survival and proliferation) and *Txn1*[346] (regulator of AP-1 signalling) are directly promoted upon SCF addition to functional HSCs (Figure 31).

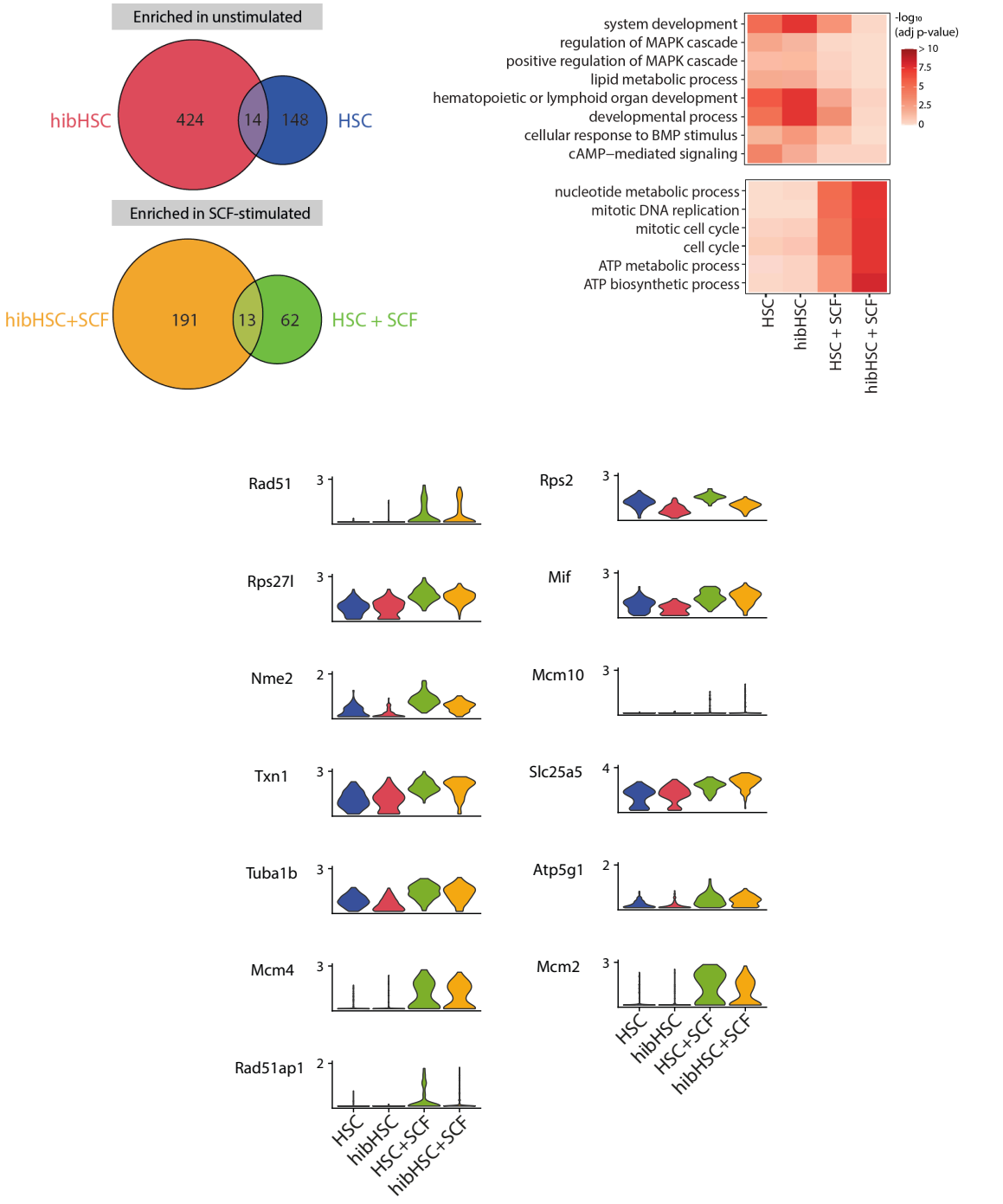


Figure 31. Changes in molecular profiles of HSC and hibHSC, upon SCF stimulation. Upper left panel: Differential gene expression (DGE) was computed for two separate comparisons: (I) comparison of fresh HSCs (HSC) against SCF-stimulated HSCs (HSC+SCF); (II) comparison of hibernating HSC (hibHSCs) against hibHSCs post SCF-stimulation (hibHSC+SCF) (negative binomial distribution, adjusted with Benjamini-Hochberg correction). Venn Diagrams represent the number of genes commonly enriched in unstimulated populations (HSC and hibHSC) and SCF-stimulated populations (HSC+SCF and hibHSC+SCF) from both separate DGE computations. Upper right panel: Gene Ontology (GO) term enrichment was computed based on differentially expressed genes, as outlined in Venn Diagrams. Minimum p-value >0.05 to be considered significantly enriched. Bottom panel: violin plots of normalised gene expression of the 13 upregulated genes in SCF-stimulated cells (HSC+SCF, hibHSC+SCF).

Interestingly, SCF stimulation did not rescue the expression of the key MolO genes downregulated in hibernating HSCs, suggesting that the reduced expression of such factors is not simply a stress-response of the cells being kept in absence of cytokine stimulation for the 7-day culture period.

Finally, hibernation cultures allowed us to identify common molecular pathways of SCF activation in purified HSCs from different physiological states.

3.9 Human HSCs can be retained as single cells in minimal cytokine culture conditions

The results so far demonstrate that SCF enhances HSC survival and proliferation *in vitro* but is not essential for HSC *in vitro* maintenance. HSCs can remain in a state of hibernation in the absence of SCF while retaining full *in vivo* functionality when transplanted. The possibility of applying these results to human HSCs could have major clinical implications, especially with respect to the possibility of maintaining and manipulating HSCs *in vitro* for stem cell transplantation, to establish protocols for *ex vivo* manipulation of HSCs and production of mature blood cells, and to study exit from quiescence.

To investigate whether minimal cytokine culture conditions would have a similar effect on human HSCs, we isolated single human $CD34^+CD38^-CD90^+CD45RA^-CD19^-CD3^-$ (hHSCs) from umbilical cord blood and cultured them in serum-free medium with human IL-11 alone for 7 days (Figure 32).

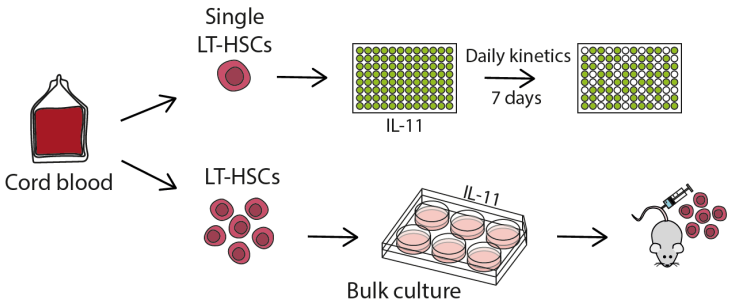


Figure 32. Schematic of single cell in vitro culture of human HSCs.
Single human HSCs ($CD34^+CD38^-CD90^+CD45RA^-CD19^-CD3^-$(LT-HSCs)) from umbilical cord blood were sorted into individual wells and cultured in presence of IL-11 with or without SCF. In parallel, human HSCs were bulk cultured for 7 days in absence of SCF and transplanted at 3 different cell doses (22, 110, and 218) into immunodeficient recipients and monitored for donor engraftment.

Similar to the mouse LT-HSCs, survival was lower with cytokine deprivation, and although some cells divided (~25.6%), a large proportion remained as single cells compared to hHSCs under standard cytokine conditions[21,238](Figure 33). The fact that some hHSCs divided may be due to the starting purity or activation state of HSCs from cord blood sources and further growth factor optimisation may permit the identification of a fully hibernating condition.

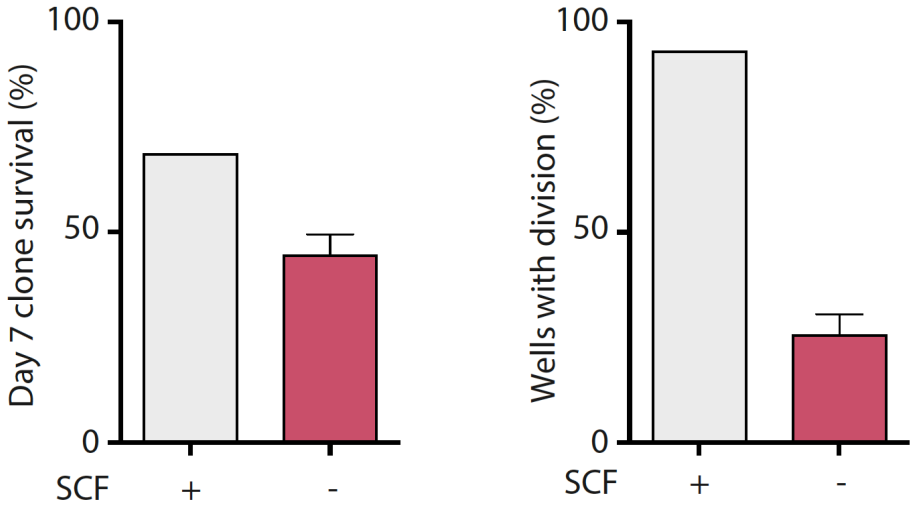


Figure 33. Hibernation conditions keep the majority of human HSCs as single cells.
The left panel shows the survival of HSCs in the presence or absence of Stem Cell Factor (SCF) over 7 days, where absence results in 1.5-fold reduced survival compared to SCF-supplemented cultures (fresh n=192, 1 biological replicate, post-hibernation n=672, 5 biological replicates). Right panel shows the proportion of cells divided after 7 days in culture with and without the addition of SCF, with the majority of cells in hibernation conditions remaining as single cells.

In order to assess the frequency of repopulating cells within the hibernating HSCs, and to assess their mature cell production *in vivo*, CB HSCs from hibernation cultures were transplanted into NSG mice. CB HSCs were bulk sorted and cultured for 7 days in the presence of IL-11 (Figure 32). Upon transplantation of limited numbers of day 7 cultured human HSCs, repopulation was stable up to 20 weeks post-transplantation, although donor repopulation was below detection for the lowest dose recipients, suggesting that limiting numbers of HSCs were retained (Figure 34). However, these results still demonstrate that IL-11 alone can maintain a proportion of multi-potent human HSCs in a non-dividing state and set the stage for further culture optimisation to support retention of larger numbers of fully functional HSCs.

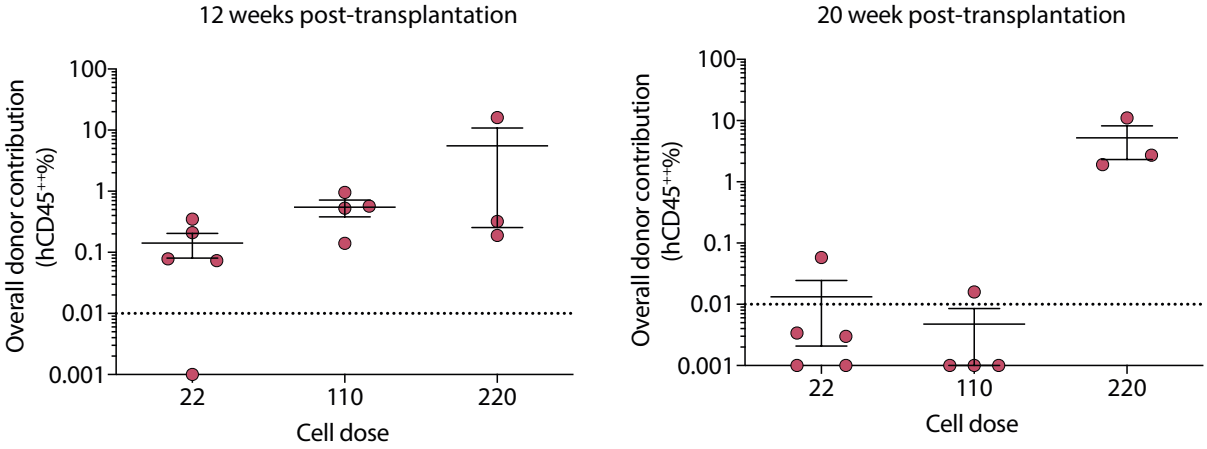


Figure 34. Overall donor contribution in NSG recipients transplanted with hibernating human HSCs.
The graphs show the percentage of human cell engraftment (%CD45^{++}) in peripheral blood from transplanted mice at 12- and 20-weeks post-transplantation (cell dose 22, n=5; 110, n=4; 218, n=3). The threshold for events considered as positive was >0.01% with a minimum of 30 analysed events. Non-engrafted mice shown below dashed line. CD45^{++}indicates cells positive for 2 distinct CD45 antibodies. Bars show mean with SEM.

Overall, minimising cytokine stimulation in the hibernation cultures allowed to isolate and maintain fully functional LT-HSCs in culture without undergoing cell division or differentiation. We were able to resolve the common molecular programmes of HSCs in different physiological states and to identify molecules that are potentially dispensable for HSC function as well as the molecular programme of SCF activation. Finally, we provided evidence that hibernating HSCs can be transduced without compromising their self-renewal ability and demonstrated the applicability of hibernation cultures to human HSCs.

4 The role of the cytokine microenvironment in myeloproliferative neoplasms

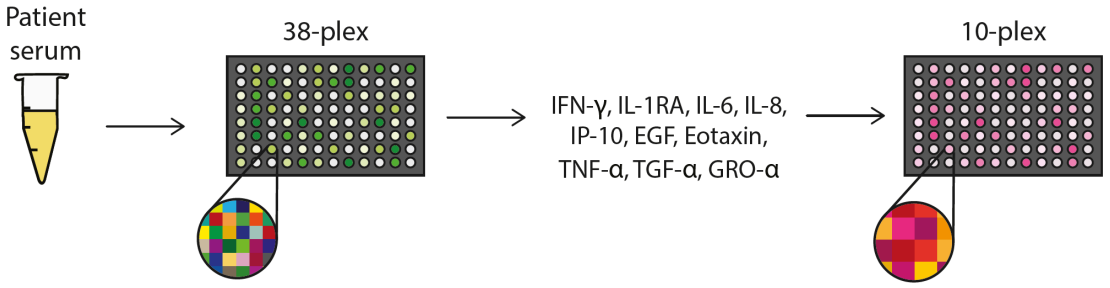
Whereas Chapter 3 focused on mouse HSCs and understanding the role of extrinsic modulators on highly purified HSCs, this Chapter attempts to ask questions about extrinsic regulators in humans, with a particular focus on how circulating cytokines contribute to disease of the haematopoietic system. While major advances have been achieved in our understanding of the cell-intrinsic regulators of haematological malignancies[347], relatively little is known about the surrounding microenvironment and what impact neighbouring cells, or the molecules they secrete, might have on disease evolution.

As described in the introduction, MPNs represent a powerful disease model for studying the early stages of tumorigenesis and key studies have suggested that the inflammatory microenvironment plays a significant role in disease establishment and maintenance[215,283,348]. Deregulation of inflammatory cytokines in MPN patients have been reported in a number of previous studies[206–208,285,286,292,293] but their exact functional role in the disease remain poorly characterised.

This Chapter focuses on the identification of inflammatory molecules that associate with MPNs and whether or not distinct cytokine patterns might correlate with different disease subtypes or disease severity. To achieve this, serum cytokine profiling was undertaken in more than 400 MPN patient samples. By combining cytokine data with clinical and genetic information, the possibility of using cytokines as potential biomarkers for disease monitoring and classification was explored and new candidates of disease modulation were revealed.

4.1 Characterisation of the MPN cytokine microenvironment across disease subtypes

In order to investigate which cytokines might play a role in the context of the MPN pathogenesis, cytokine concentration of 38 different inflammatory molecules were initially measured in serum samples from 185 MPN patients, to identify those that were most discriminatory for MPN subtype (Figure 35). The screen covered a selection of cytokines and chemokines involved in well-characterised inflammatory pathways and patient serum samples were taken at diagnosis or shortly thereafter in order to reduce the potential interference of treatment associated factors, such as anti-inflammatory therapies. The concentration of each molecule was measured by using an ELISA-based immunoassay which allowed quantification of each selected molecule in the same sample simultaneously.



Cohort	Number of patients	Male (%)	Hb (g/dL)	WBC (x10^9/L)	Plt (x10^9/L)	sMF	sAML	Deaths	Follow-up (years)
ET	146	46	138	8.4	804	6	2	7	5.2 (0.7-21.4)
PV	94	61.1	177	10.8	385	1	0	7	5.2 (1.0-28.4)
MF	51	62.5	114	8.8	340	-	4	11	4.8 (1.0-25.3)
ET (PT-1)	122	42.3	136	9.6	983	27	26	62	11.9 (0.2-43.6)

Figure 35. Serum profiling of cytokines and chemokines in MPN samples.
Overview of serum cytokine screen. Levels of 38 cytokines were assessed in serum samples from MPN patients by multiplexed ELISA. Following data analysis, 10 cytokines were selected for their ability to track with disease subtype, disease severity or overall survival. The table on the bottom shows the high-level summary data for patient groups. Median follow-up time is displayed in years with minimum/maximum follow-up time indicated in parenthesis. ET=essential thrombocythaemia, PV=polycythaemia vera, MF=primary myelofibrosis, Hb=haemoglobin, WBC=white blood cell count, Plt=platelet count, sMF=secondary MF, sAML=secondary AML. Median values are displayed for Hb, WBC and Plt.

A high degree of heterogeneity was observed in serum levels of most cytokines across the patient cohort, indicating substantial patient-patient variability (Figure 36).

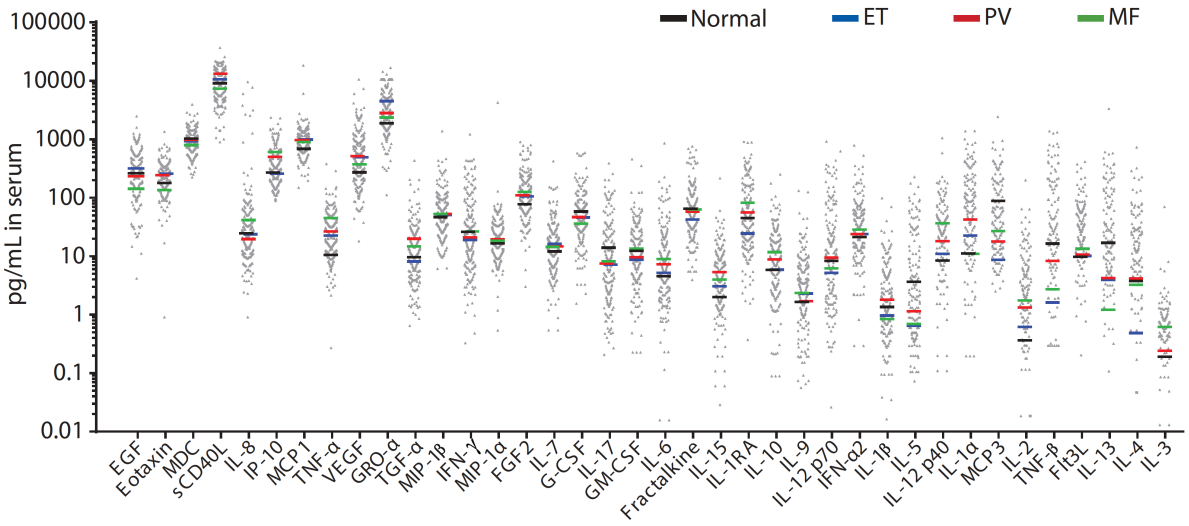


Figure 36. Cytokine screen of 38 cytokines and chemokines in 190 MPN diagnostic samples.
Serum concentration (pg/mL) of 38 inflammatory cytokines and chemokines across 190 MPN patients and 11 normal individuals (grey triangles). Bars show medians for each MPN subtype and controls (n=11 normal, black line; n=107 ET, blue line; n=52 PV, red line; n=31 MF, green line). ET=essential thrombocythaemia, PV=polycythaemia vera, MF=myelofibrosis.

Despite this, the analysis identified several differences in cytokine levels across MPN subtypes with a number of cytokines significantly altered in specific patient groups, showing strong disease specificity. Each of TGF-α, IP-10, IL-8, IFN-γ, IL-1RA, GRO-α, EGF and Eotaxin (CCL1) were significantly different for at least one MPN subtype. High levels of IP-10 and TNF-α were significantly associated with MF, the most severe MPN subtype compared to control non-MPN and ET patients. The opposite trend was observed for Eotaxin and EGF which were both associated with the less severe subtypes PV and ET compared to MF. Similarly, GRO-α was significantly increased in ET patients (Figure 37).

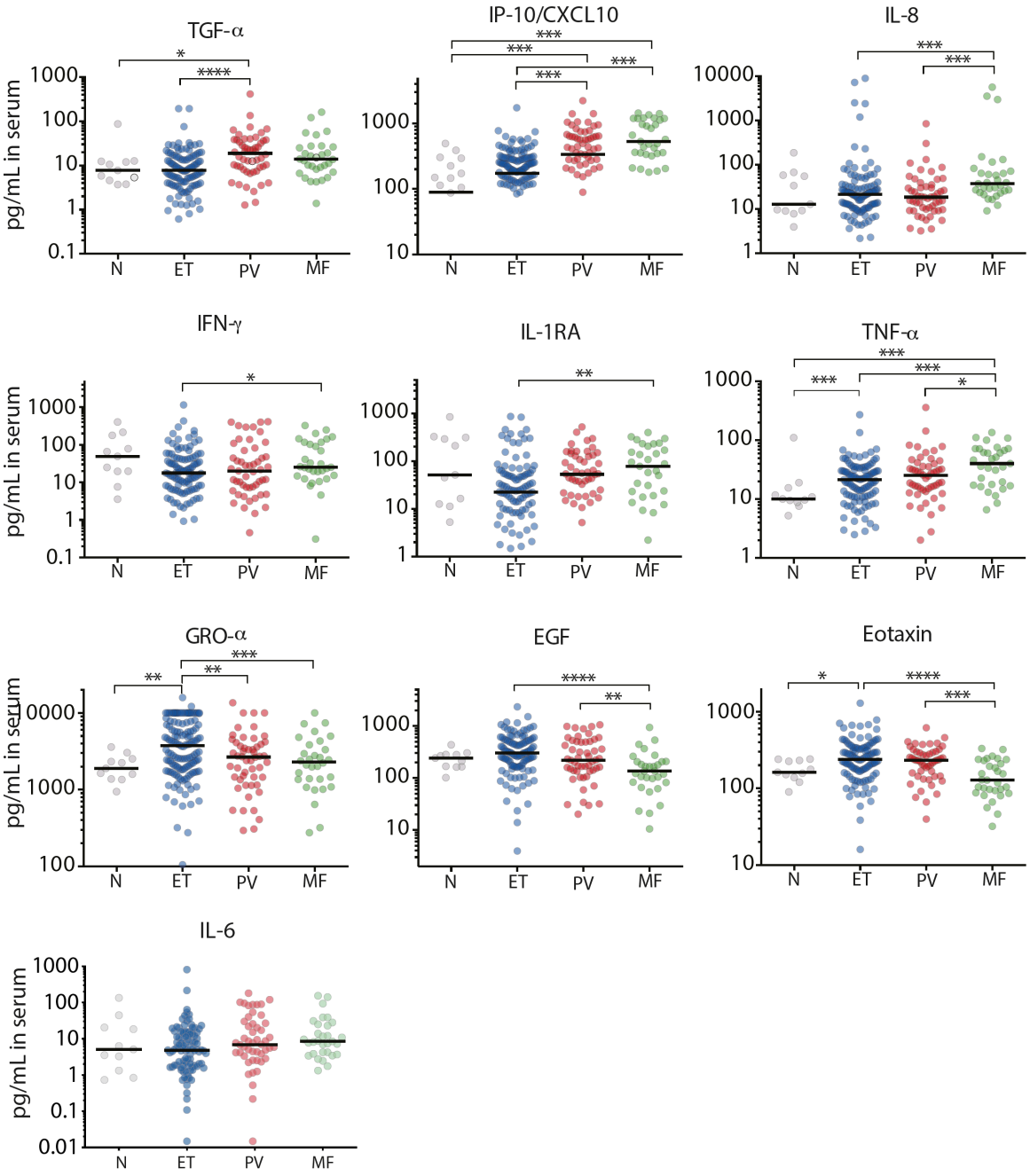


Figure 37. Serum levels of 10 selected cytokines in the 38-plex screen.
Comparisons of the serum levels of 10 selected cytokines across the 190 MPN patients and 11 normal individuals. Bars show medians. Mann-Whitney U-test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N=normal, ET=essential thrombocythaemia, PV=polycythaemia vera, MF=myelofibrosis.

Based on their association with disease severity (e.g., TNF-α) and/or disease specificity (e.g., GRO-α), we selected 10 informative cytokines and designed a custom 10-plex assay in order to further explore their association with disease pathogenesis and progression. The 10 selected cytokines, a summary of their function, and their

MPN-related feature are listed in Table 5. IFN-γ levels were higher in MF compared to ET patients, but not significantly different between MPN patients and healthy controls. However, it was included in the screen because of previous evidence showing a putative role in MPN pathogenesis[349]. IL-6 levels did not differ across different MPNs, but its levels were significantly associated with patient survival across all MPN subtypes.

Table 5. Cytokine candidate list.
List of the 10 selected candidates from the 38-plex screen, their key function and the MPN-related differences.

Cytokine		Function	MPN-related feature
IP-10	Interferon-induced protein-10	Chemotaxis (mono/macro/T/NK), apoptosis, cell growth angiostasis	↑ in PV, MF vs ET, healthy controls
IL-8	Interleukin-8	Chemotaxis	↑ in MF vs PV
EGF	Epidermal growth factor	Cell growth and differentiation	↑ in ET, PV vs MF
Eotaxin		Chemotaxis (neutrophils)	↑ in ET, PV vs MF
TGF-α	Transforming growth factor alpha	Proliferation, differentiation and development	↑ in PV vs ET
IFN-γ	Interferon gamma	Activation of macrophages, NK, and neutrophils	Previously implicated in MPN biology[349]
GRO-α	Gro alpha	Chemotaxis, angiogenesis, mitogenesis	↑ in ET vs PV, MF, healthy controls
IL-1RA	Interleukin-1 receptor antagonist	Proliferation, differentiation and cell survival	↓ in ET vs healthy controls
TNF-α	Tumour necrosis factor	Pro-inflammatory cytokine	↑ in MF vs ET, healthy controls
IL-6	Interleukin-6	B-cell growth, antagonistic to regulatory T cells	Differences in patient survival across MPN subtypes

The 10-plex array was first validated by screening the same samples previously run on the 38-plex array, and then comparing the data generated with the data obtained from the previous screen, to determine whether the level of multi-plexing impacted the data outcome. When plotting the values for each cytokine obtained for the 10-plex and the 38-plex against each other, each of the 10 cytokines showed good correlation (R^2 values ranging from 0.1-0.69), confirming that the reduced complexity of the panel did not significantly affect the assay readout (Figure 38). The divergence that did exist, was largely due to values that fell outside the standard curve range of the

assay (3.2-10,000 pg/mL): GRO-α levels exceeding a concentration of 10,000 pg/mL were depicted at a maximum value of 10,000 to ensure compatibility between samples. Low levels of IFN-γ, IL-1Rα, IL-6, and EGF were also poorly correlated between the 10-plex and 38-plex protein assay and was possibly due to protein levels being below the detection limits of the kit.

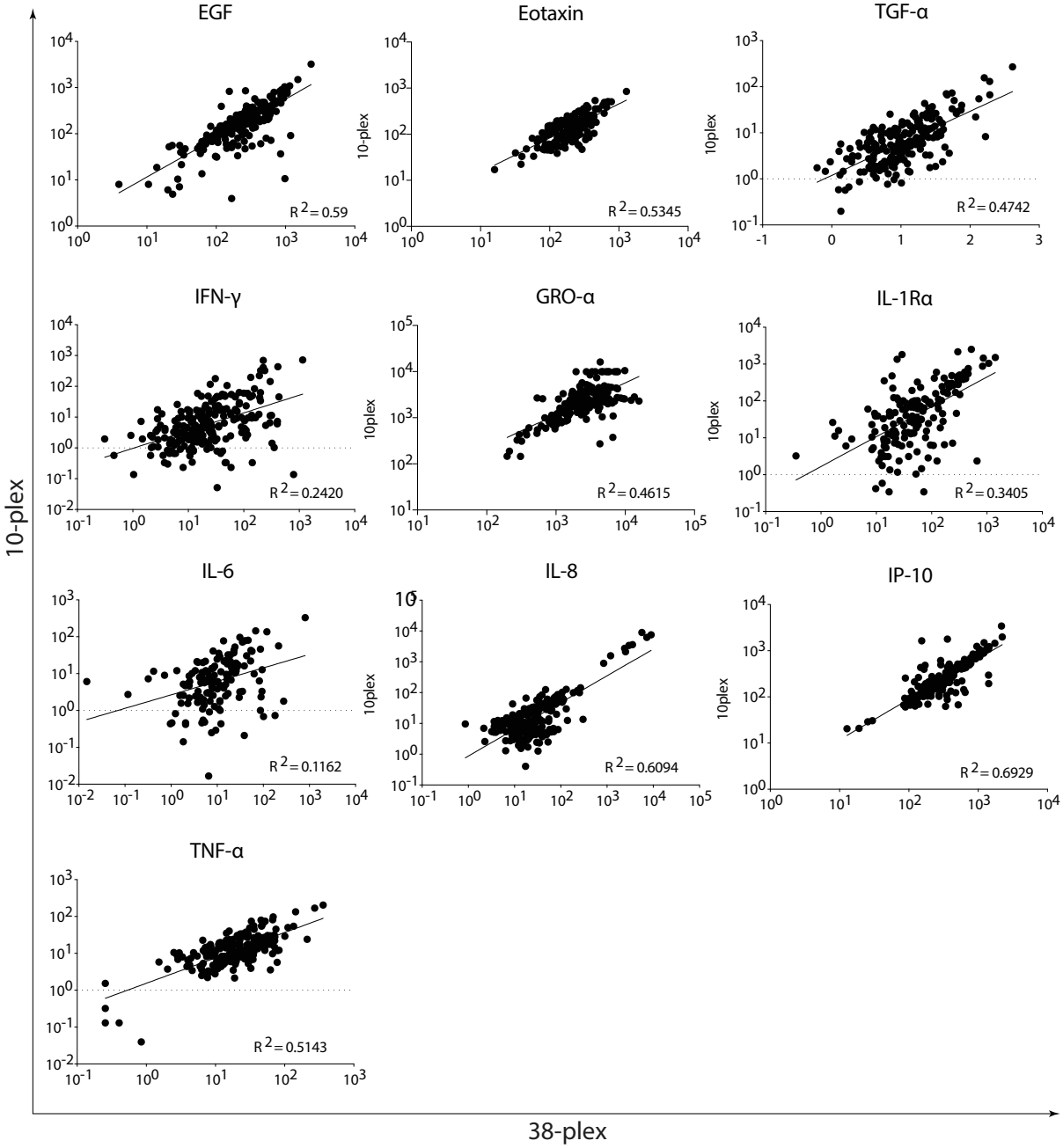


Figure 38. Validation of the 10-plex assay and comparison to the 38-plex array.
All 10 cytokines show good correlation (R^2 value ranging from 0.1-0.69) when compared to the original 38-plex. Poor correlation is observed when values fall below standard curve range (3.2-10,000 pg/mL).

In order to validate distinct cytokine profiles of disease subtype, new patient serum samples were collected (n=291) and screened for the 10 selected cytokines (Figure 35). The analysis compared patients diagnosed with ET (n=146), PV (n=94) and MF (n=51) to each other and to healthy controls (n=14) in order to identify cytokines that track with disease severity and subtype. The highlights of the screen are discussed in the following sections.

4.2 Myelofibrosis is associated with an inflammatory environment

Myelofibrosis has been previously described as an inflammatory disease[204]. Among the MPN subtypes, it is considered the most advanced disorder, with the most severe clinical features, including BM fibrosis, cytopenia and extramedullary haematopoiesis, and the highest tendency toward leukaemic transformation, along with variable constitutional symptoms (e.g., fever, fatigue), all of which have a primary impact on the quality of life. These distinct clinical features have been partly attributed to the overproduction of inflammatory cytokines; however, the specific role of individual cytokines has had limited investigation in MF patients.

In accordance with these observations, our data show a significant increase of a number of prototypical inflammatory cytokines in MF patients, compared to PV, ET and healthy controls. Particularly striking examples were the increased levels of IP-10 and IL-8 compared to normal controls and to ET patients and the significant increase in TNF-α compared to both normal controls and to ET and PV subtypes (Figure 39).

Taken together, these results confirmed previously published work which suggested that increased production of inflammatory cytokines is a recurrent, and potentially pathophysiologic, feature of MF[206,207,285,350] and set the stage for further investigating the exact role of inflammatory cytokines in the context of disease establishment or evolution.

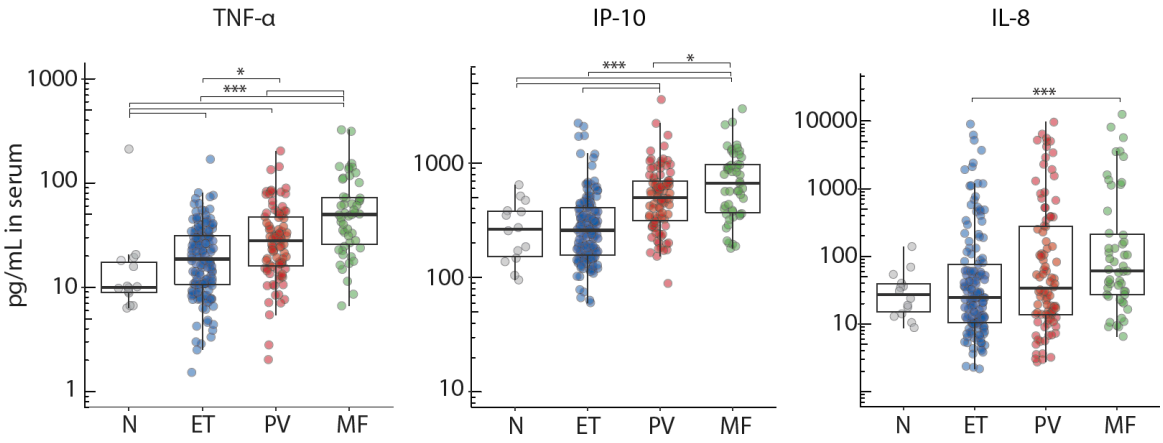


Figure 39. Increased levels of TNF-α, IP-10 and IL-8 in patients with MF.
Levels of individual cytokines increased in serum of patients with myelofibrosis, compared to the other MPN subtypes and healthy controls. Graphs displaying data from ET (n=146), PV (n=94), MF (primary myelofibrosis, n=51) and age-matched normal controls (N, n=14). Boxes show medians with interquartile range (IQR). Mann-Whitney U test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N=normal, ET=essential thrombocythaemia, PV=polycythaemia vera, MF=myelofibrosis.

4.3 A cytokine signature for essential thrombocythemia

Within the MPNs, ET are considered the least severe subtype with the longest life expectancy and most manageable symptoms. Previous studies have concluded that relatively lower levels of pro-inflammatory cytokines and chemokines were present in patients with ET compared to those with PV and MF[204]. In agreement with this, the majority of inflammatory cytokines were expressed at low levels in our initial 38-plex screen, including those involved in adaptative immune activation and chemo-attraction (e.g., IFN-γ and IP-10). These data, together with the low levels of IL-6 and TGF-α measured in ET compared to controls and other MPN subtypes, support the notion that ET patients do not have a particularly high amount of circulating inflammatory cytokines (Figure 36).

However, our data also indicate a number of instances of significant up-regulation of cytokines in ET patients. The levels of GRO-α were markedly raised in ET patients compared to other MPNs, while EGF and Eotaxin were higher in both ET and PV (i.e., chronic phase of disease) compared to the more advanced disease stage of MF (Figure 40). Together, these results show that a specific subset of inflammatory cytokines are

associated with the ET subtype, and different inflammatory cytokine levels might also be predictive for the least severe subtype.

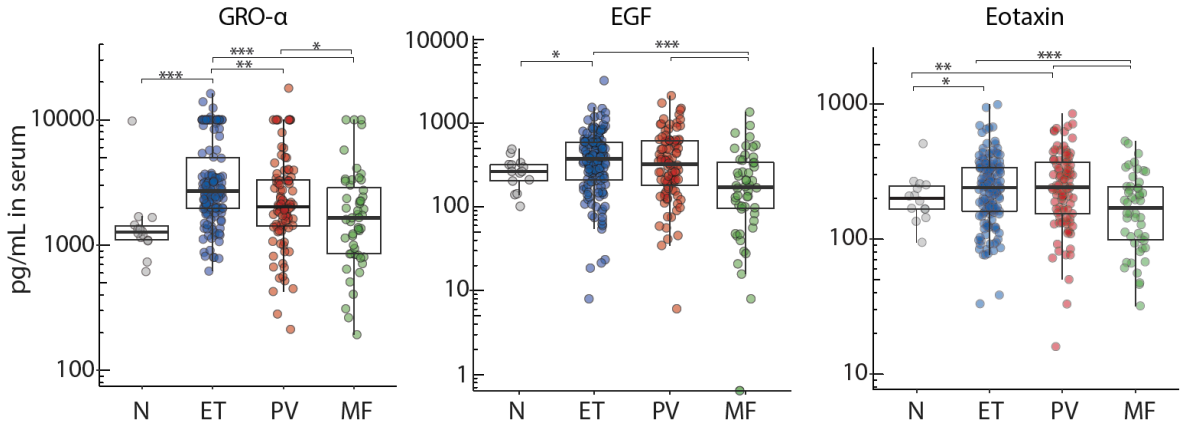


Figure 40. Increased levels of GRO-α, EGF and Eotaxin in patients with ET.
Levels of individual cytokines that are increased in serum of patients with ET, compared to other MPN subtypes and healthy controls. Graphs displaying data from ET (n=146), PV (n=94), MF (primary myelofibrosis, n=51) and age-matched normal controls (N, n=14). Boxes show medians with interquartile range (IQR). Mann-Whitney U test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. N=normal, ET=essential thrombocythaemia, PV=polycythaemia vera, MF=myelofibrosis.

4.4 GRO-α levels in serum are predictive of transformation in ET

ET is associated with haemorrhage, thrombosis and risk of transformation to MF and AML. Given the higher expression of a specific set of inflammatory molecules in ET, we decided to further explore whether these inflammatory factors correlated with the ET subtype, by determining whether levels of any cytokine(s) might be predictive of disease evolution and/or transformation to more severe disease. Long-term clinical outcome data was available for 182 of the 291 ET patients in the original cohort.

The data in ET patients (n=116) showed that, among the cytokines measured, high levels of GRO-α correlated with increased risk of transformation from ET to MF over 15 years (Figure 41, left panel). However, since our initial cohort only had a small number of transformation events (n=8, 6 secondary MF (sMF), 2 secondary AML (sAML)), we extended our analysis of the 10 cytokine levels to a selected cohort of ET patients from the PT1 trial[324–326], where genomic and clinical characterisation was available with a median of 11.9 years of follow-up (range 2 months to 43 years from

diagnosis). From the >1200 patients in the PT-1 trial, the patient cohort we selected was highly enriched for transformation events, with 26 sAMLs, 30 sMFs and 69 deaths. In this analysis, samples were diluted 1:5 as it was observed that in some cases, levels of GRO-α exceeded the range covered by the standard curve. The correlation of high GRO-α levels with MF-transformation risk was confirmed in this dataset (Figure 41, right panel). Altogether these results suggest that measuring levels of GRO-α in serum from ET patients at diagnosis can be informative for predicting disease outcome. Moreover, because diagnostic MF samples did not report increased GRO-α levels (Figure 40), our data suggest that high levels of GRO-α are exclusively associated with ET subtype and they might be able to serve as potential predictive markers of future chronic phase transformation rather than simply a hallmark feature of MF.

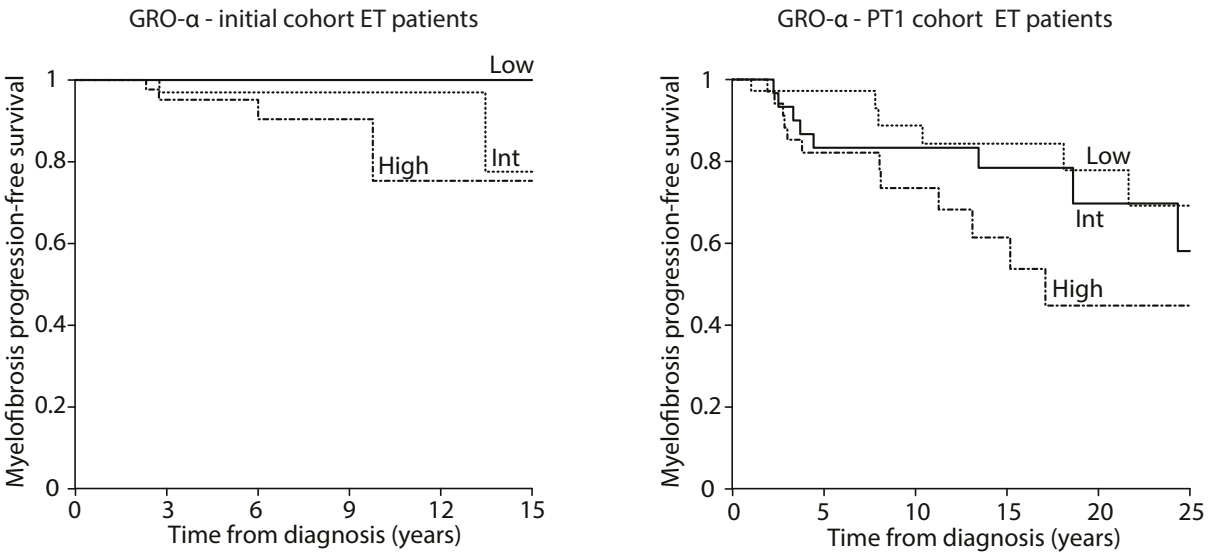


Figure 41. GRO-α levels in diagnostic samples are predictive factor of transformation in ET patients.
Left panel: Kaplan-Meier analysis of progression-free survival according to pre-transformation levels of GRO-α in ET and PV patients with MF-transformation follow-up data in initial cohort (n=151). High levels of GRO-α correlated with increased risk of transformation from chronic phase (ET or PV) to sMF (Cox proportional hazards modelling including age, sex and diagnosis, p=0.004). Right panel: Kaplan-Meier analysis of progression-free survival in PT-1 cohort. High levels of GRO-α correlate with increased risk of transformation from ET to sMF (Cox hazards modelling including age and sex, p=0.01). For both initial cohort and PT-1 cohort, patients have been stratified into equally sized groups.

4.5 Longitudinal profiling of EGF levels is predictive of transformation events

The vast majority of previous serum cytokine studies only assessed a single time point, thereby providing a static picture of the cytokine environment at a certain stage of the disease. Sampling at multiple intervals could be useful to track consistency in cytokine levels over time and also to eventually normalise the measurement for daily physiological fluctuation in protein levels in the blood. Moving in this direction, we decided to extend our study to longitudinal samples to determine whether any of the chronic phase markers (EGF, Eotaxin, and GRO-α) might be useful for disease monitoring and for predicting the risk of subsequent transformation. We observed that the rate of EGF level change strikingly correlated with transformation risk, with the vast majority of transformations (~80%) observed in patients whose EGF levels decreased over time. Stability was defined as an absolute rate of change of <8pg/mL per year. This value was an arbitrary assignment based on splitting the patient cohort into relatively equal groups. Using a lower cut-off of 4pg/mL was also significant for predicting transformation.

In comparison with patients with stable or increasing EGF levels, there was a 4.3-fold (95% confidence interval 1.7-10.9) increased likelihood of transformation to MF or AML if EGF levels reduced over the course of the longitudinal sampling, strongly indicating the utility of monitoring EGF levels during the course of the longitudinal profiling (Figure 42). Correction for baseline reticulin grade and treatment with anagrelide or hydroxycarbamide did not alter the correlation between decreasing EGF levels and risk of myelofibrotic transformation, confirming the potential utility of monitoring EGF levels over the course of the disease.

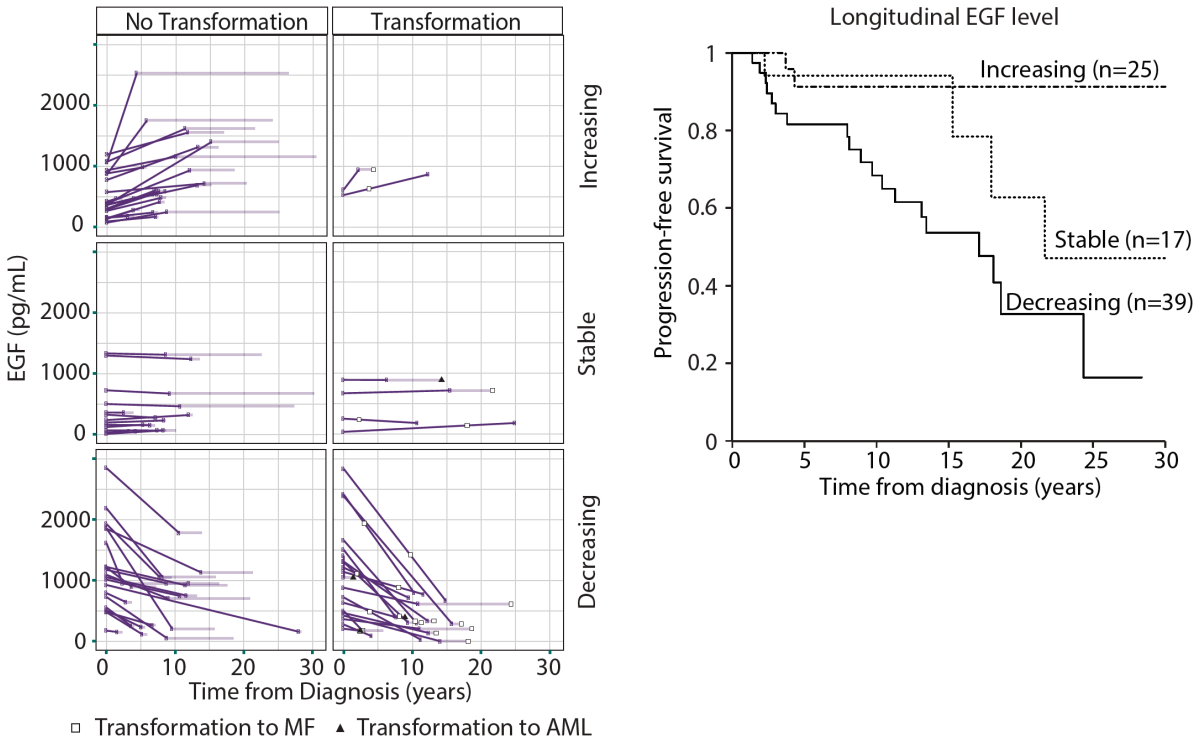


Figure 42. Decreases in EGF serum levels over time associate with disease transformation

Left panel: serum EGF levels were assessed at multiple time points during disease and patients were classified as having increasing, decreasing, or stable levels of EGF, where EGF stability was defined as an absolute rate of change of <8pg/mL per year. 18/39 (46%) patients with decreased levels of EGF over time transformed to MF or AML, while only 4/17 (24%) and 2/25 (8%) of patients with stable and increasing levels of EGF respectively transformed to MF or AML. Longitudinal samples taken pre- or post-transformation are separated accordingly. Dark lines refer to the point up until sampling occurred while light lines indicate non-sampled follow-up. Right panel: Kaplan-Meier curve of progression-free survival displaying patients cohorts defined by increasing, stable or decreasing EGF levels over time. Patients with decreasing levels of EGF were 4.3-fold (95% confidence interval 1.7-10.9) more likely to transform to MF or AML (p-value=0.008). MF=myelofibrosis, AML=acute myeloid leukaemia.

4.6 Cytokine measurements improve on genomics for predicting transformation-free survival

We next assessed the predictive yield (as assessed by model concordance, equivalent to the area under the curve of the receiver-operator characteristic) of adding cytokine quantification to prognostic models utilising demographic and clinical data alone, and those additionally incorporating genomic variables. Concordance statistic are often used to assess the ability of one or multiple risk factors to predict outcome[351]. In our model, we assessed age, sex, levels of all 10 cytokines and the presence/absence of 33

genetic mutations for their capacity to predict transformation to sMF or sAML and survival in MPN patients (Figure 43, upper panel). This analysis confirmed a recently published study showing that comprehensive genomic characterisation improved the prediction for disease transformation[328], and demonstrated that this could be further improved by measuring cytokine levels. High GRO-α levels, as well as high IL-8 and IP-10 levels, were predictive for MF transformation, independent of age, sex, haematological and genomic parameters, with a GRO-α level greater than the median associated with a hazard ratio of 4.1 (95% confidence interval 1.6-10.4) for transformation (Figure 43, bottom left panel). Levels of IL-8, GRO-α and IP-10, in combination with the other informative statistics (age and presence/absence of an SRSF2/U2AF1 mutation) were integrated in a multivariate prognostic model with quartiles of 30 patients each, defined by their calculated risk and how further patient stratification. Inclusion of cytokine levels improved the performance of the prognostic model, as assessed by the Concordance-statistic for goodness of fit, confirming that cytokine profiling improves on clinical data for predicting transformation-free survival (Figure 43, bottom right panel).

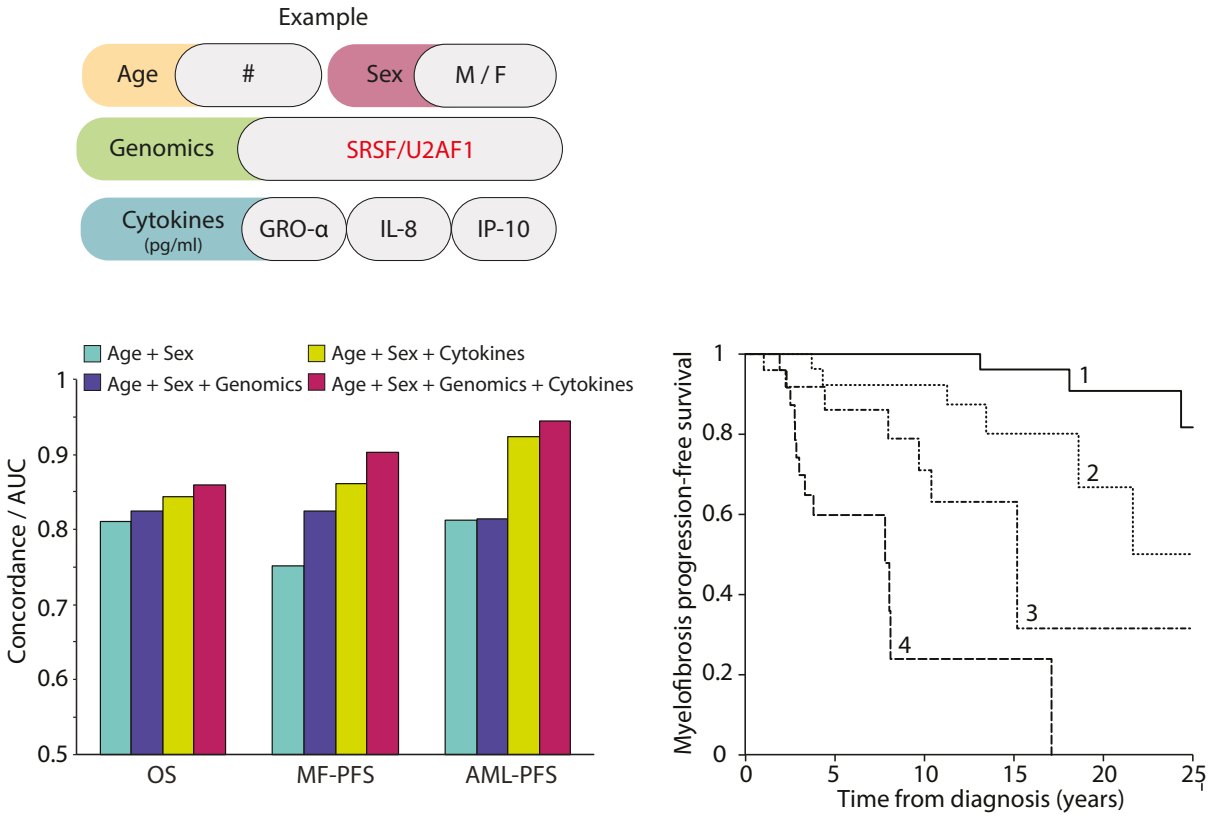


Figure 43. Cytokine profiling add prognostic value beyond genomics data alone. Upper schematic: variables considered in the prognostic model: age, sex, presence/absence of 33 driver mutations, level of 10 cytokines. Bottom left panel: the predictive yield (equivalent to the area under the curve for the receiver-operator characteristics, assessed by model concordance) of adding cytokine quantification to prognostic models based on demographic and clinical data alone, and to those additionally incorporating genomic variables. Inclusion of cytokine measurements further improves power of genomic characterisation in predicting disease transformation. OS=overall survival; MF-PFM=myelofibrosis progression-free survival; AML-PFS=AML progression-free survival with transformation follow-up data (n=122). Bottom right panel: Kaplan-Meier curve of progression-free survival displaying patients stratified into equally sized group (quartiles of 30 patients each) according to their predicted risk, as defined by a multivariate Cox proportional hazards model (using age, GRO-α levels, IL-8 levels, IP-10 levels, and presence/absence of an SRSF2/U2AF1 mutation ($p<0.001$)).

Altogether, these results highlight the importance of combining biological information with genetic and clinical dataset to improve disease stratification and to predict patient outcomes. In addition, monitoring cytokine levels at diagnosis and during the course of disease will be important to define which inflammatory factors play a role in the context of MPNs and detail how MPN-associated inflammatory cytokines might contribute to disease evolution.

5 The role of the IP-10 in myeloproliferative neoplasms disease severity

As described in Chapter 4, our cytokine profiling identified a number of inflammatory molecules which were differentially upregulated across different MPN disease subtypes. In particular, the cytokine that most strongly correlated with disease severity was IP-10 (or CXCL10), whose levels were significantly elevated in myelofibrosis patients compared to healthy controls and the chronic phase disease subtypes (ET, PV). Therefore, we elected to follow up the clinical and genomic data related to IP-10 and to undertake a complementary cytokine screening and functional assays in mouse models of MPNs.

This Chapter aims to delineate the role of IP-10 in MPN pathogenesis. In order to investigate whether IP-10 is required for the disease initiation and maintenance, mouse models with robust and trackable phenotype (the JAK and JAK TET double mutant mice) were used to assess the ability of MPN cells to initiate and/or sustain disease in an IP-10 deficient microenvironment.

5.1 IP-10 levels correlate with JAK2 and TET2 mutational status

In Chapter 4, it was reported that a number of cytokines analysed in the serum screen correlated with a specific MPN disease subtype. Comprehensive data on mutation status for genes commonly mutated in MPNs, as well as recurrent chromosomal abnormalities (including JAK2, CALR, MPL, TET2, DNMT3A, ASXL1, EZH2, SRSF2, 9pUPD, and del(20q)) was available for the majority of patients described in Chapter 4[328]. We therefore asked whether the presence of specific mutations correlated with the level of specific cytokines. All patients were characterised for both JAK2V617F status and collaborating mutations by genetic sequencing or exome sequencing, and clinical data were collected when available (e.g., age, sex, etc.).

After correction for diagnosis, age and sex, few differences in cytokine levels were observed across patients based on mutational status alone, including those with no detected mutations, suggesting that a single genetic lesion or combination of lesions do not seem to dominantly instruct micro-environmental heterogeneity. The single interesting exception was IP-10, which showed a positive correlation with JAK2V617F variant allele fraction in ET, PV and MF patients (Figure 44A). When additional mutations were considered in combination with JAK2V617F homozygosity, patients who carried mutations in epigenetic regulators (TET2, DNMT3A, ASXL1, EZH2, or IDH1/2, JAK2hom+) had the highest levels of IP-10 in all disease subtypes when compared to patients with JAK2-heterozygous (JAK2het), CALR, or MPL mutations (Figure 44B), suggesting that the larger and more advanced MPN clones might drive higher production of IP-10.

Most strikingly, when all MPN subtypes were combined and patients were sub-grouped based on JAK2V617F mutational status, those with JAK2V617F homozygosity showed higher levels of IP-10 compared to JAK2V617F-negative patients. Importantly, homozygous patients for the JAK2V617F mutation who also carried loss of function mutations in TET2, the gene most commonly co-mutated with JAK2, reported the highest levels of IP-10 (Figure 44C). Altogether, these observations suggest that the microenvironment differences observed across disease subtypes

could be correlated to the genetic background and that mutations in JAK2 and TET2 might be responsible for the up-regulation of IP-10 in serum from MPN patients.

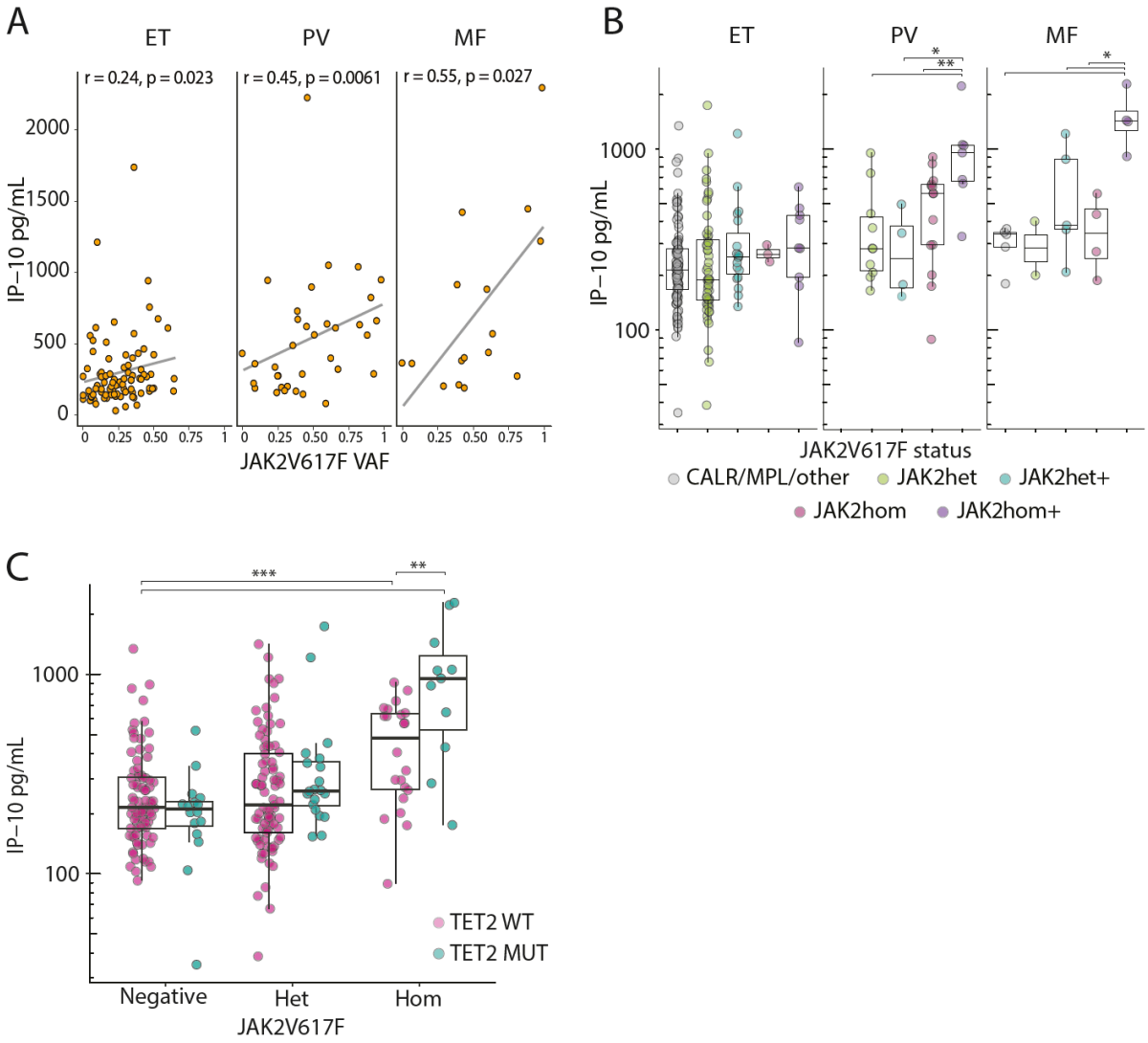


Figure 44. Serum levels of IP-10 correlate with JAK2 and TET2 mutational status in MPN patients.
A) Correlation of IP-10 serum levels with JAK2V617F burden in each disease subtype. High levels of IP-10 correlate with an increase in JAK2V617F allele burden. B) Serum IP-10 levels in ET, PV and MF patients (JAK2-mutation negative (CALR/MPL/other), grey circles; JAK2V617F heterozygous alone (JAK2het), green circles; JAK2het with an additional epigenetic mutation (JAK2het+), blue circles; JAK2V617F homozygous alone (JAK2hom), pink circles; JAK2hom with an additional epigenetic mutation (JAK2hom+), purple circles. JAK2hom+ patients reported the highest levels of IP-10 in both the PV and MF subgroups. C) IP-10 serum levels in patients with JAK2 and TET2 mutations. JAK2V617F-positive patients (Hom) have increased levels of IP-10 compared to non-mutant patients (Negative). The highest levels of IP-10 are detected in patients with both JAK2 homozygosity (Hom) and TET2 mutations (TET2 MUT). Bars show medians with IQR. Mann-Whitney U test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ET=essential thrombocythaemia, PV=polycythaemia vera, MF=myelofibrosis.

5.2 IP-10 levels are also raised in MPN mouse models

We next investigated whether the consequences of these changes could be accurately modelled in mice by profiling a similar panel of inflammatory cytokines in 4 MPN mouse models (JAK2V617F KI (JAK)[256], TET2 KO (TET)[249], CALR Del5 (CALR)[263], JAK2 TET2 double mutant (JAK TET)[262]). Serum samples were collected and screened for 32 different cytokines and chemokines, in order to identify potential differences across different mutation models and to explore potential overlap in differential expression across different disease phenotype, highlighted in the human screen.

An overview of the screen is displayed in Figure 45. Most of the factors screened were not detected at significantly different levels across the different models or compared to WT control mice, potentially suggesting that disease models and WT mice present common microenvironmental factors and/or that such factors are not primarily involved in the disease. A few cytokines were not detectable, probably due to their levels being below the detection limits of the kit (e.g., IFN-γ).

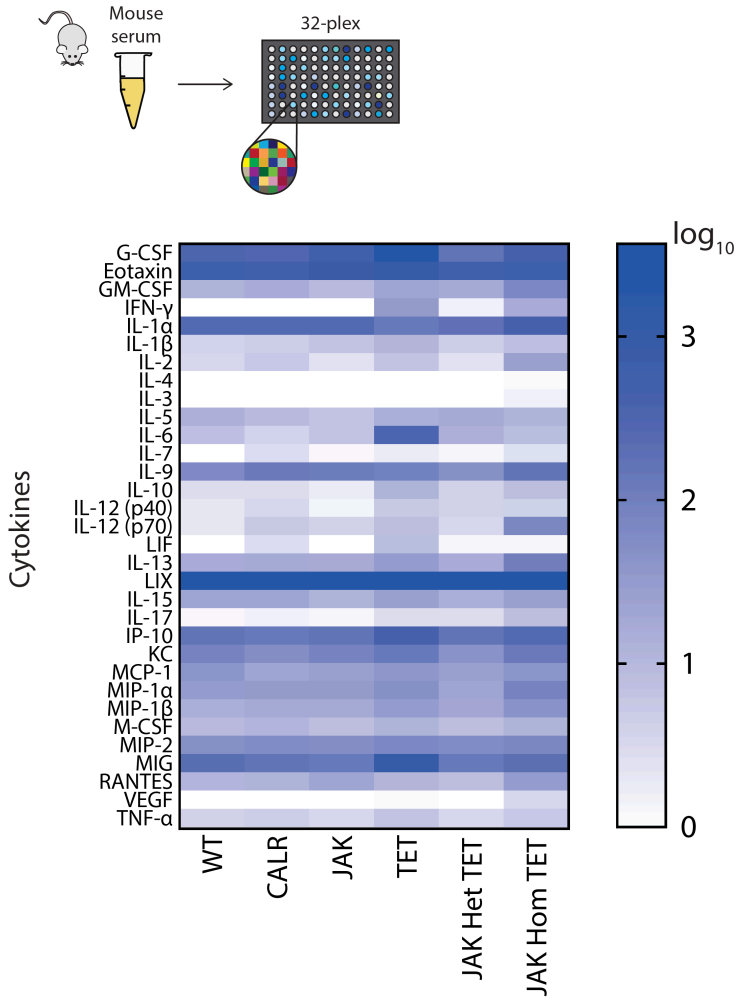


Figure 45. Cytokine screen of serum samples from MPN mouse models.
Levels of 32 cytokines were assessed in serum samples from MPN mouse models by multiplex ELISA (wild type (WT), n=9; CALR Del5 (CALR), n=7; JAK2V617F KI (JAK), n=5; TET2 KO (TET), n=11; JAK Het TET, n=12; JAK Hom TET, n=10). Heatmap displays serum concentration (pg/mL) mean for each genotype. TET, JAK Het TET, and JAK Hom TET groups include both heterozygous and homozygous mice for TET mutation, as no significant difference for each of the cytokine levels was observed between TET Hom and TET Het mice.

Again, as in the human data, the one cytokine which was interesting and showed a strong correlation with genotype and disease severity was IP-10. Serum levels of IP-10 were elevated in TET mice compared to WT mice, with even higher increases observed in JAK Hom TET double mutant mice (Figure 46). As for most of the other cytokines screened, CALR mutant mice did not show any significant increase in IP-10 levels, which were comparable to the WT mice.

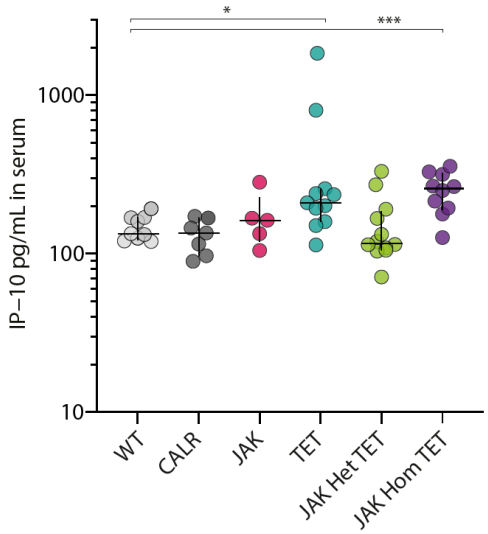


Figure 46. IP-10 levels in mouse models of myeloproliferative neoplasms.
Serum levels of IP-10 in wild type (WT, n=9), CALR Del5 (CALR, n=7), JAK2V617F KI (JAK, n=5), TET2 KO (TET, n=11), JAK Het TET (n=12), and JAK Hom TET (n=10) mice. Serum levels of IP-10 are increased in TET and JAK Hom TET mice, compared to WT control mice. Bars show median in IQR. Mann-Whitney U-test * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

These data strongly indicate that these specific mutations alter cytokine levels and by extension the BM microenvironment in which the disease-driving cells exist. Importantly, these results suggested that the JAK TET series of MPN mouse models could be an effective tool to further explore and characterise the impact of the microenvironment, and in particular of IP-10, on disease establishment and/or progression.

5.3 IP-10 does not have a direct effect *in vitro* on single HSCs

The observation that levels of IP-10 in serum associated with the MF subtype and significantly correlated with JAK2 and TET2 mutational status in both MPN patients and the JAK TET mouse models suggests that IP-10 might play a role in the disease development, maintenance or evolution. This makes it important to explore its functional role in disease biology, specifically whether or not IP-10 acts directly on stem and progenitor cell expansion which would subsequently lead to disease outgrowth. In order to assess the direct biological impact of IP-10 on mutant stem cells, single HSCs were isolated from MPN patient samples and MPN mouse models, cultured in the presence or absence of IP-10 and assessed for cell division and survival after 10 days of *in vitro* culture. IP-10-treated HSCs did not show a significant

difference in survival compared to the untreated counterparts. Moreover, no differential effect of IP-10 was observed across the different MPN subtypes or MPN mouse models (Figure 47).

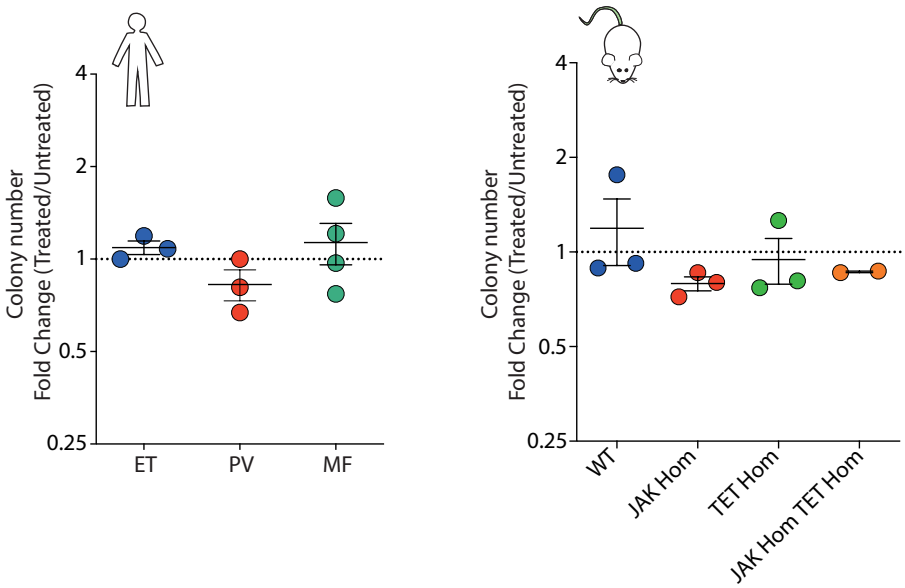


Figure 47. *In vitro* effect of IP-10 on HSC survival in MPN patients and mouse models. Single HSCs were sorted from MPN patients (ET, n=3; PV, n=3; MF, n=4) and from mouse models of MPNs (WT, n=3; JAK Hom, n=3; TET Hom, n=3; JAK Hom TET Hom, n=2), cultured for 7 days with (Treated) or without (Untreated) IP-10 (50ng/mL and 100 ng/mL for human and mouse HSCs respectively) and assessed for cell survival. Treated HSCs do not show significant differences in survival over the untreated controls. Bars show mean with SEM. ET=essential thrombocythemia, PV=polycythaemia vera, MF=myelofibrosis, WT=wild type, JAK Hom=JAK2V617F KI, TET Hom=TET KO, JAK Hom TET Hom= (JAK2V617F KI/TET2KO).

These results suggest that IP-10 may not have a direct effect on mutant and normal HSCs *in vitro*, although the assay is limited in length and assessment of other HSC functional activities beyond survival. If HSCs do not respond to IP-10 stimulation *in vitro*, it is possible that IP-10 could be having an indirect effect by modulating different cell types or their relative proportions and then exerting an effect on HSCs.

5.4 Absence of IP-10 does not prevent JAK2-TET2 double mutant HSCs from driving an MPN in IP-10 KO recipients

Although treatment with IP-10 does not directly affect mutant HSC survival *in vitro*, its role in the context of the *in vivo* microenvironment remains unclear. IP-10 KO mice were therefore acquired to determine its role in the context of the MPN initiation or

evolution[312]. IP-10 deficient mouse do not have a severe phenotype, but do show impaired T-cell function, including a defect in T-cell proliferation and recruitment of effector T-cells in response to specific antigenic challenges, such as stimulation with OVA (ovalbumin)[312]. After obtaining the IP-10 KO model, we first validated the absence of IP-10 by PCR (Figure 48) and confirmed that no previously undiscovered haematopoietic phenotype that might interfere with the MPN features, was present in the stem/progenitor and mature cell compartments.

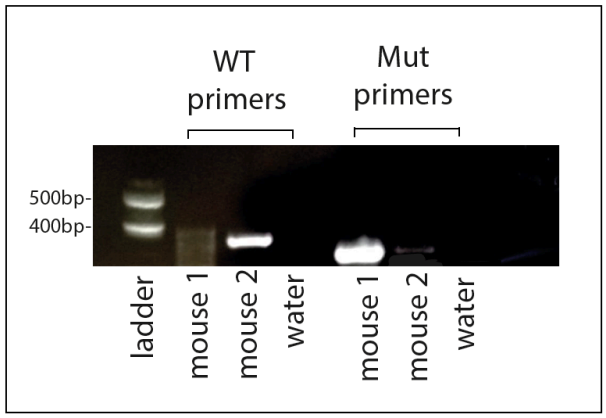


Figure 48. Representative image of IP-10 KO genotyping experiment.
Primers for both the wild-type and mutant IP-10 are listed in Methods. Mice were genotyped based on the presence/absence of each band. In this representative image, mouse 1 is a mutant IP-10 KO and mouse 2 is a WT mouse. 100kb ladder on the left.

To do this, we first surveyed the BM of IP-10 and WT mice to determine the cellular composition of stem/progenitor cells (ESLAM and LSK) and mature cells (T-, B-, and myeloid cells). IP-10 KO and WT mice have similar lineage composition in the BM and no significant differences were observed in LT-HSCs and LSK proportions (Figure 49).

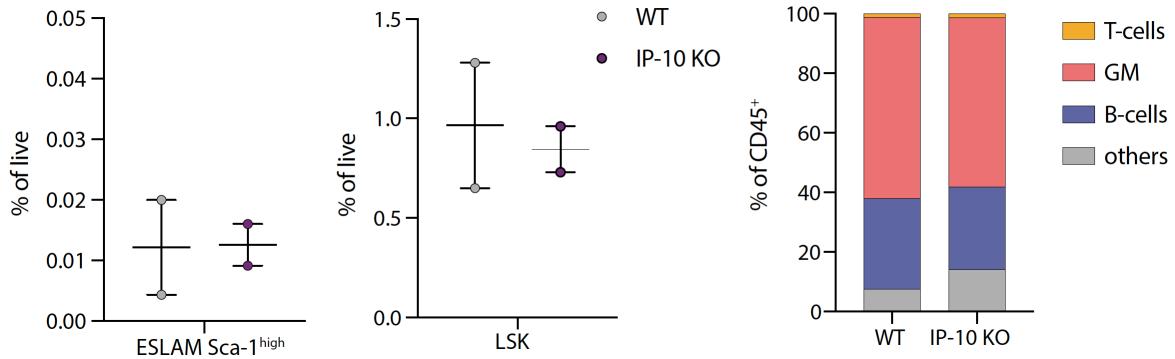


Figure 49. Representative comparison between WT and IP-10 KO mouse BM composition.
Left panel: graph shows percentage of ESLAM Sca-1^{high} (CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca-1^{high}) and LSK (Lin^{-}Sca-1^{+}c-Kit^{+}) BM cells from WT (n=2) and IP-10 KO (n=2) mice. No significant differences in ESLAM Sca-1^{high}(p=0.9423) and LSK (p= 0.6560) proportions are observed between WT and IP-10 KO. Paired t-test. Right panel: graph shows percentage of T-, B-, and GM in in the CD45^{+} cell fraction in WT (n=1) and IP-10 KO (n=1) mice. Cells were classified as T-cells (positive for CD3 marker), B-cells (positive for B220 marker), and GM (positive for CD11b and Ly6g (myeloid cells marker)), CD3 (T-cell marker), and B220 (B-cell marker).

In order to investigate whether the absence of IP-10 affects HSC function *in vitro*, single ESLAM Sca-1^{high} HSCs were isolated from WT and IP-10 KO mice and cultured *in vitro* for 10 days, tracking divisional kinetics and clone survival. Time to first, second, and third division of IP-10 KO HSCs overlapped nearly identically with WT HSCs (Figure 50, top). Clone survival and clonal proliferation over the 10-day culture was also highly similar to WT mice, as measured by assessing clone size distribution, suggesting that there were no major perturbations of HSC function *in vitro* due to the absence of IP-10 alone (Figure 50).

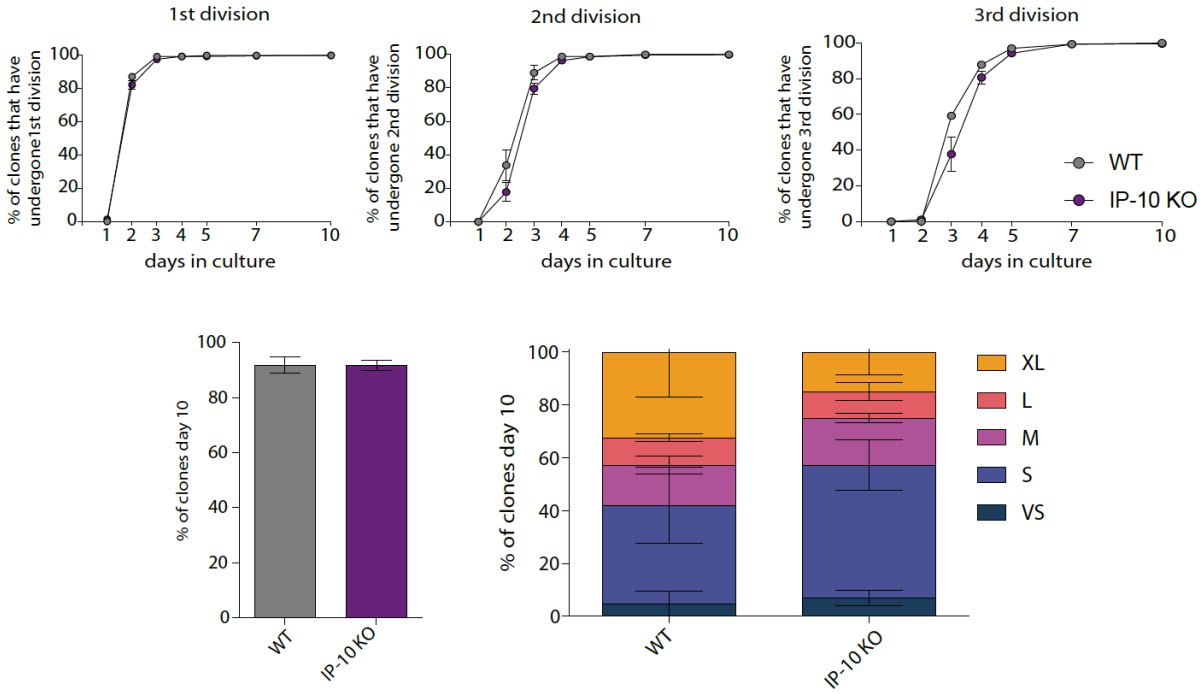


Figure 50. Loss of IP-10 does not alter proliferation of HSCs *in vitro*.
Single CD45^{+}EPCR^{+}CD48^{-}CD150^{+}Sca-1^{high} (ESLAM Sca-1^{high}) LT-HSC from IP-10 KO (3 biological replicates) and WT mice (2 biological replicates) were sorted into individual wells of 96 well plates, cultured for 10 days in StemSpan with 10%FCS, 300ng/mL SCF, and 20ng/mL IL-11, and assessed for proliferation, cell cycle kinetics, and survival. Top panels: IP-10 KO HSCs (purple line) do not have altered cell cycling compared to WT (grey line) HSCs. Two-way ANOVA (Sidak's multiple comparisons test). Bottom left panel: IP-10 KO (purple bar) and WT (grey bar) HSCs have comparable clone survival at day 10 post-isolation (Unpaired t-test, p=0.9801). Bottom right panel: colony size was measured on day 10 post-isolation and no differences in clone size distribution was observed between IP-10 KO and WT HSCs. Two-way ANOVA (Sidak's multiple comparisons test): VS=very small, p=0.9999; S=small, p=0.7776; M=medium, p=0.9998; L=large, p>0.9999; XL=very large, p=0.5105. All bars show mean with SEM.

Once it was established that the number and *in vitro* function of HSCs from IP-10 KO was highly similar to WT HSCs, the next experiment was to investigate whether a robust disease could be established and maintained in an IP-10-deficient environment. To do this, HSCs were isolated from mice with a robust MPN phenotype and transplanted into IP-10 KO mice (Figure 52). For this, the JAK2V617F KI / TET2 KO double mutant mouse (JAK TET) were selected[262]. The JAK TET mice have a robust, serially transplantable disease, resembling the human PV subtype, with haematocrit (HCT) levels greater than 80%. Transplantation of BM cells from JAK TET mice into WT mice also enables the establishment of an MPN disease (PV-like phenotype), indistinguishable from the disease of the donor (Figure 51).

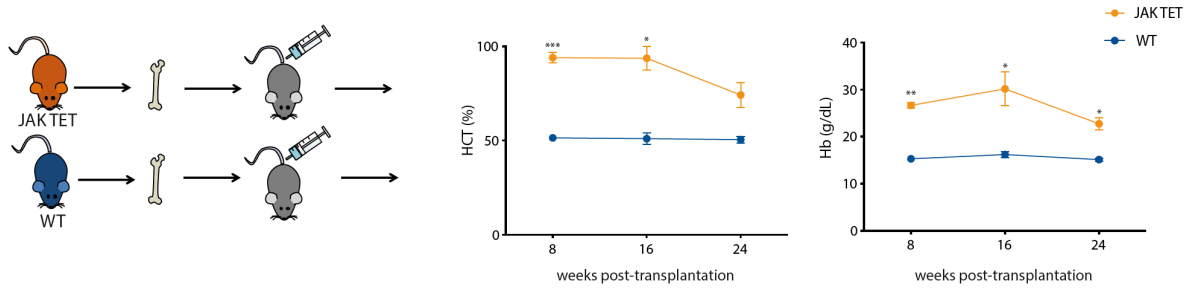


Figure 51. The JAK TET mouse model possesses a robust, transplantable PV-like disease.
JAKV617F KI/TET2 KO (JAK TET) mice exhibit a PV-like disease subtype with high haematocrit and haemoglobin levels. Transplantation of BM cells from JAK TET mice into WT mice recreates the same disease into the recipients (data in the graph adapted from Shepherd et al.,2018[262]). Bars show mean with SEM. HCT=haematocrit, Hb=haemoglobin.

In order to test whether the IP-10 present in the host microenvironment is essential for disease initiation and whether its depletion has any effect on the transplanted disease, JAK TET HSCs were transplanted into an IP-10-deficient background. 100 ESLAM LT-HSCs (25 cells/mouse) were therefore isolated from JAK TET mice and transplanted alongside with IP-10 KO helper cells (300,000 cells/mouse) into WT or IP-10 KO recipient mice. Recipients were serially bled at 4, 8-, 12-, 16- and 20-weeks post-transplantation and peripheral blood cell counts were performed to monitor HCT, Haemoglobin (Hb), platelet (Plt), and white blood cell (WBC) count (Figure 52A).

Results in Figure 52B show that both WT and IP-10 KO recipients show an increase in HCT and Hb levels, resembling the donor PV-like phenotype, and demonstrate that absence of host-derived IP-10 does not prevent JAK TET HSCs from initiating an MPN in a transplantation setting. IP-10 KO and WT recipients show comparable Plt levels at all time points. At 12 weeks post-transplantation, a slight increase in HCT and WBC was observed in IP-10 KO mice compared to the WT recipients, and WT recipients show higher Hb at week 8 post-transplantation.

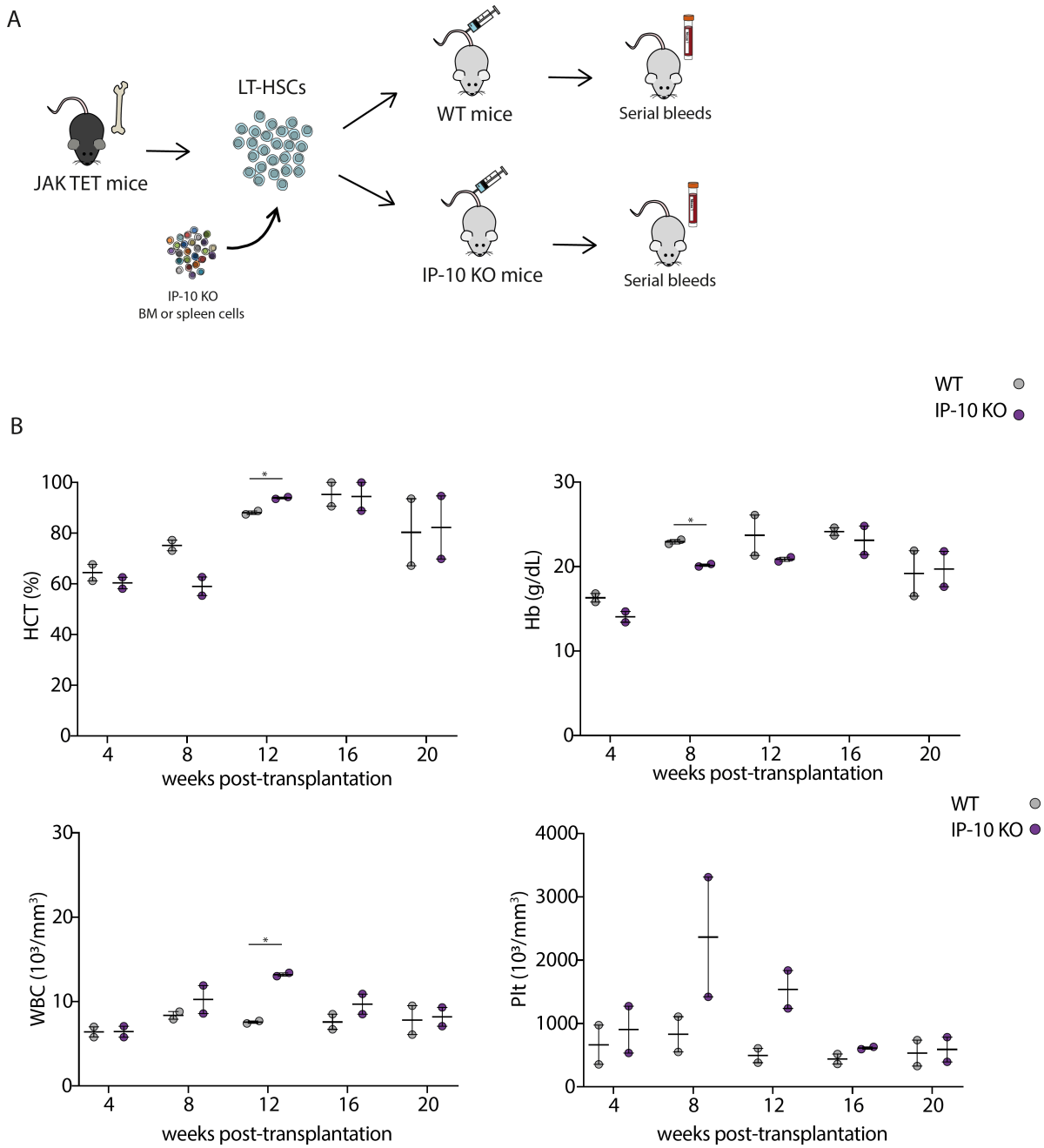


Figure 52. IP-10 is not required for development of the disease in a murine transplantation model of MPN.

A) Transplantation of JAK TET cells into IP-10 KO or WT recipients. Bulk HSCs (25 per mouse) were sorted from JAK TET (JAK2V617F KI/ TET2KO) mutant mice and transplanted into either WT (n=2) or IP-10 KO (n=2) recipients, along with BM cells from IP-10 KO ("helper cells", 300,000 per mouse). Serial bleeds were taken at week 4, 8-, 12-, 16-, and 20-weeks post-transplantation and peripheral blood cell parameters analysed. B) Haematocrit (HCT), haemoglobin (Hb), white blood cell (WBC), platelets (Plt) count for IP-10 KO and WT recipients transplanted with the same donor cells. No significant differences observed in Plt at any time point. A slight increase in HCT and WBC is reported in IP-10 KO mice compared to WT recipients (p=0.0186 and p=0.002 respectively) at week 12 post-transplantation, and in Hb levels in WT mice compared to IP-10 KO, at week 8 post-transplantation (p=0.0103). Bars show mean with SEM. Unpaired t-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Similar conclusions could be drawn when HSCs from JAK-mutant mice were transplanted into IP-10 KO recipients (Figure 53A). IP-10 KO and WT recipients exhibit the same PV-like phenotype, with increase in HCT and Hb over the first 16 weeks post-transplantation. A slight reduction of these HCT and Hb values is observed at week 24, possibly due a reduction of the JAK2 clone expansion, as a consequence of a limited number of HSCs transplanted (Figure 53B).

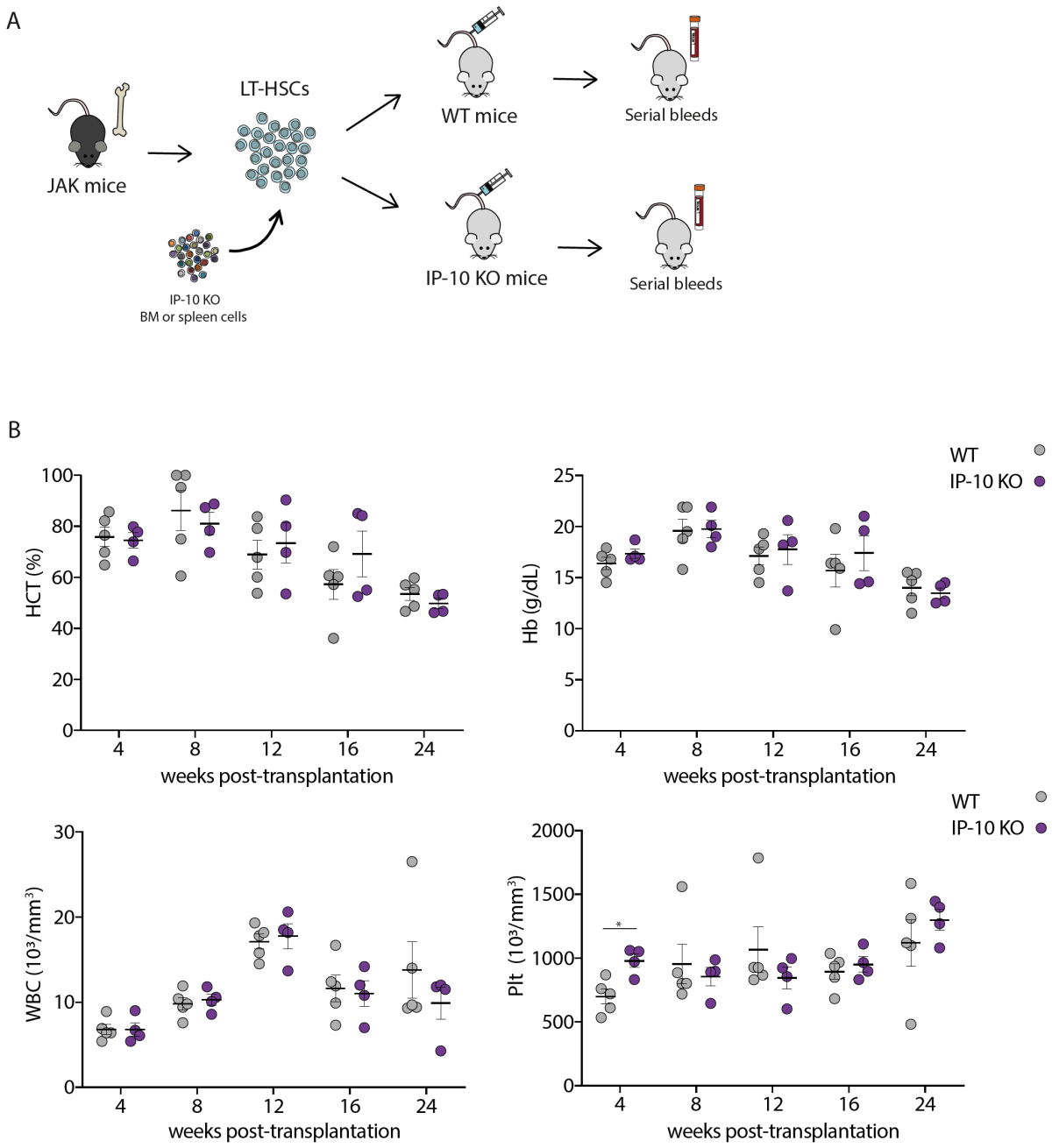


Figure 53. JAK2-mutant cells can recreate the disease when transplanted into IP-10 KO recipients.

A) Transplantation of JAK Hom (JAK2V617F KI mouse) cells into IP-10 KO or WT recipients. Bulk HSCs (25 per mouse) were sorted from JAK Hom mutant mice and transplanted into either WT (n=5) or IP-10 KO (n=4) recipients, along with BM cells from IP-10 KO ("helper cells", 300,000 per mouse). Serial bleeds were taken at week 4, 8-, 12-, 16-, and 20-weeks post-transplantation and peripheral blood cell parameters analysed. B) Haematocrit (HCT), haemoglobin (Hb), white blood cell (WBC), platelets (Plt) of IP-10 KO and WT recipients transplanted with the same donor cells. A slightly significant increase in Plt is observed in IP-10 KO compared to WT recipients, at week 4 post-transplantation (p= 0.0103) No significant differences observed at each time point. Bars show mean with SEM. Unpaired t-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Together, these results indicate that the removal of IP-10 from the host microenvironment does not prevent the initiation of disease when either JAK or JAK TET HSCs are transplanted into IP-10 KO mice. This could be due to the mutant donor cells creating their own IP-10 as the clone grows which may help maintain the disease. The mild reduction in HCT and Hb levels in the early stages of the purified JAK TET HSC transplantation experiments is also concordant with this possibility, where HSCs would not be expected to have generated large numbers of mature IP-10 secreting cells at early time points.

5.5 Combinatorial loss of IP-10 with a JAK2V617F mutation does not result in a significant change in PV-like phenotype

Since transplantation of JAK TET HSCs might lead to the donor mutant cells (and/or their progeny) producing IP-10 and re-introducing the cytokine into the environment, we next undertook genetic crosses to assess the consequences of complete absence of IP-10. IP-10 KO mice were crossed with the JAK2V617F KI line in order to generate a model that would completely remove IP-10 from a JAK2-mutant background in order to test whether its presence was essential to developing the disease phenotype. Hereby I will refer to these mice as JAK IP-10 double mutant mice.

Peripheral blood was collected and analysed from 16-week-old JAK IP-10 double mutant mice. A mild decrease in Hb levels was observed in JAK IP-10 mice (both IP-10 Het and IP-10 Hom) compared to the JAK Hom mice, suggesting that loss of IP-10 might partially ameliorate the phenotype, however, HCT levels were still high in both models.

In agreement with previous data, a reduction in platelet counts was observed in the JAK Hom mice, and this was also the case for the JAK IP-10 double mutant mice compared to WT controls (Figure 54). Finally, in agreement with the original Li et al. publication[256], JAK Het mice displayed a moderate, although not significant, increase in white blood cell counts compared to WT mice. The increase was less significant in JAK Het IP-10 Het and JAK Het IP-10 Hom mice, suggesting that loss of IP-10 might partially restore the alteration in WBC levels.

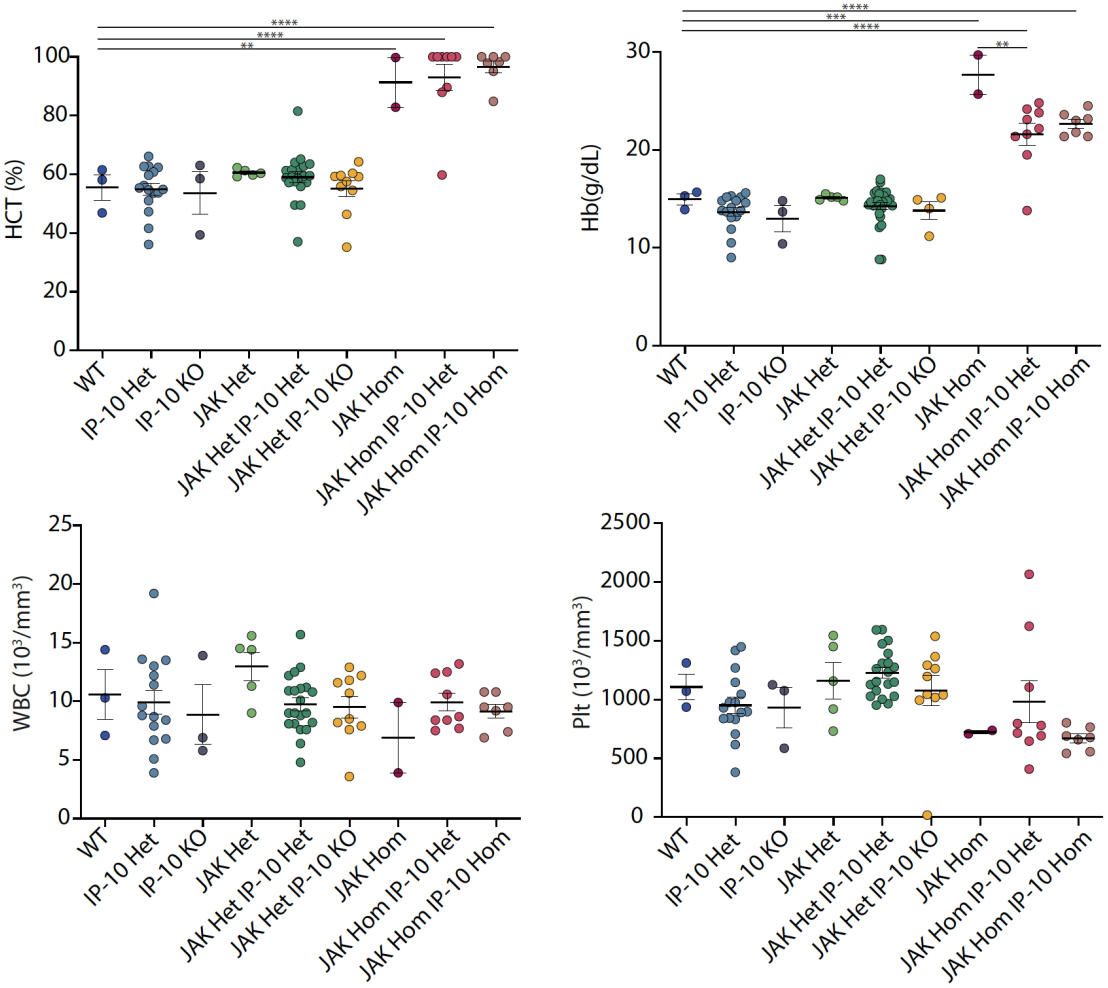


Figure 54. IP-10 loss does not prevent the disease to development in JAK2-mutant mice.

16-week-old mice were bled, and blood cell counts were performed. The graphs show haematocrit (HCT), haemoglobin (Hb), white blood cell (WBC), and platelet (Plt) counts. JAK Hom, JAK Hom IP-10 Het, and JAK Hom IP-10 Hom show increased HCT ($p=0.0010$, $p<0.0001$, $p<0.0001$ respectively) and Hb ($p<0.0001$, $p=0.0002$, $p<0.0001$ respectively) compared to WT mice. A slight reduction in Hb levels was observed in JAK Hom IP-10 Het and JAK Hom IP-10 Hom compared to JAK Hom mice ($p=0.0085$ and $p=0.0740$). JAK Het IP-10 Het and JAK Het IP-10 KO mice show a mild decrease in WBC compared to JAK Het mice ($p= 0.4258$ and $p=0.4642$ respectively). One-way ANOVA (Tukey's multiple comparisons test): *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Bars show mean and SEM.

5.6 Loss of IP-10 in the JAK2V617F KI model causes a mild reduction in terminally differentiated erythroblasts

As previously reported by Li et al.[256], the JAK2-mutant mice (JAK Hom) present a PV-like phenotype, displaying marked increase in haematocrit, splenomegaly, and increased erythroid and megakaryocytic hyperplasia in BM and spleen. To investigate

whether their cellular composition was altered by the absence of IP-10, BM and spleen cells from 16-week-old JAK IP-10 mutant mice were isolated and analysed for mature/progenitor cell composition by flow cytometry. Representative FACS profiles of the gating strategies are shown in Figure 11 of the Methods.

In the BM, a mild relative decrease in the B-cell fraction in JAK Hom, JAK Hom IP-10, and in GM fraction in JAK Hom, was observed when compared to WT mice. In the spleen, the loss of IP-10 did not induce any significant differences in the proportion of LSK or $CD3^+$ populations in the JAK IP-10 cross, when compared to the JAK mice controls (Figure 55).

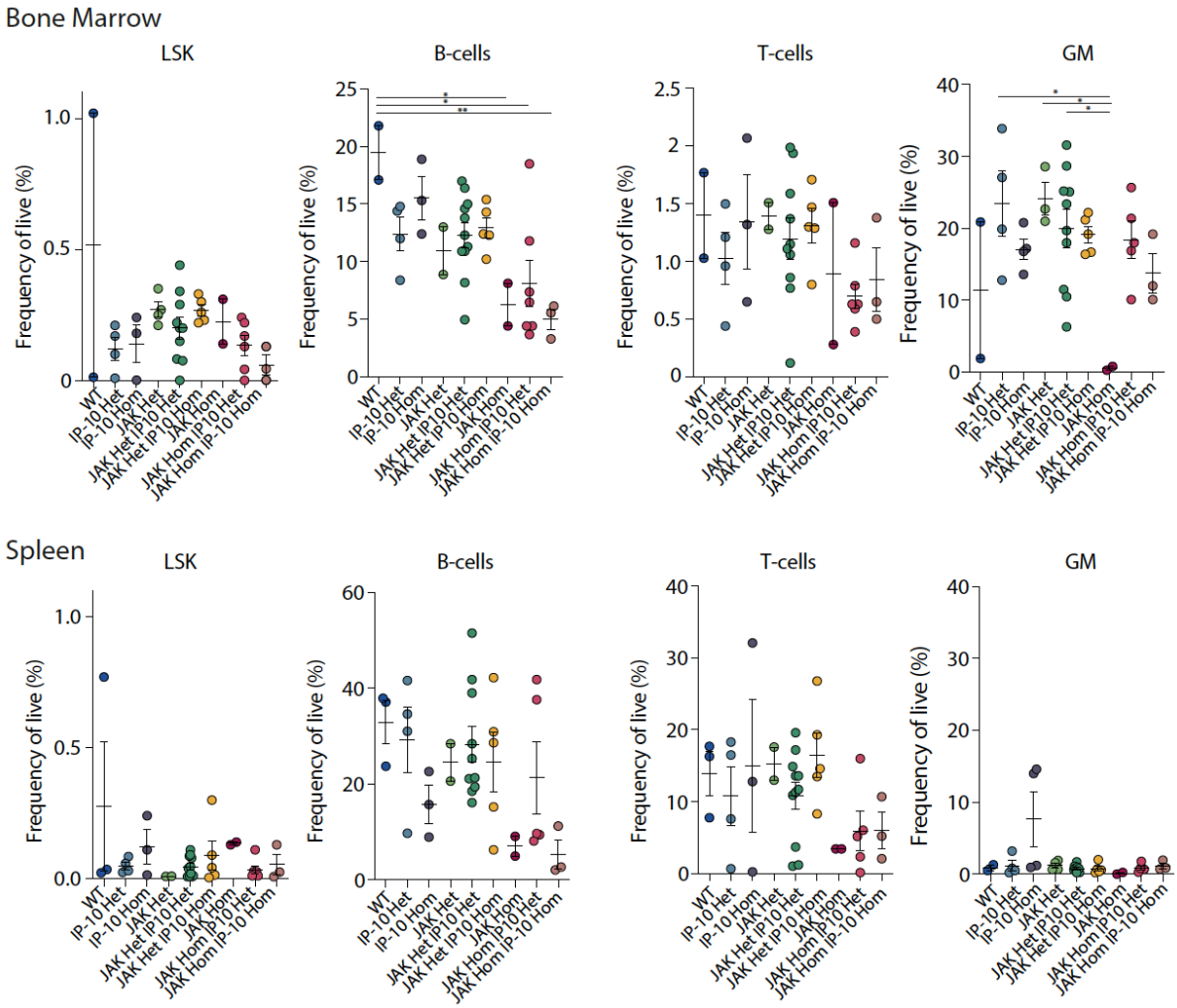


Figure 55. BM and spleen mature cell composition within JAK IP-10 mice.
BM and spleen cells from JAK IP-10-mutant mice were isolated and mature cell composition analysed (WT, n=2; IP-10 Het, n=4; IP-10 Hom, n=3; JAK Het, n=2; JAK Het IP-10 Het, n=11; JAK Het IP-10 Hom, n=5; JAK Hom, n=2; JAK Hom IP-10 Het n=5; JAK Hom IP-10 Hom, n=3). Graphs show the percentage of LSK ($Lin^{-}Sca1^{+}c\text{-}kit^{+}$), $B220^{+}$ cells (B-cell marker), $CD3^{+}$ (T-cell marker), $CD11b^{+}Mac\text{-}1^{+}$ (GM markers) in viable BM cells. JAK Hom, JAK Hom IP-10 Het, and JAK Hom IP-10 Hom show a slight decrease in B-cells and GM in the BM, when compared to WT mice. One-way ANOVA (Tukey's multiple comparisons test): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Bars show mean with SEM.

Next, since JAK Hom mice have a striking erythrocytosis, the mature erythroid compartment was assessed. In accordance with previously reported data, the proportion of stage V terminally differentiated erythroblast ($CD71^{-}Ter119^{+}$) was increased in JAK Hom mice compared to WT mice[255]. A slight, although not significant, reduction in this population fraction was observed in JAK Hom IP-10 mice, when compared to the JAK Hom, hinting that red cell maturation may be influenced by the lack of IP-10 (Figure 56).

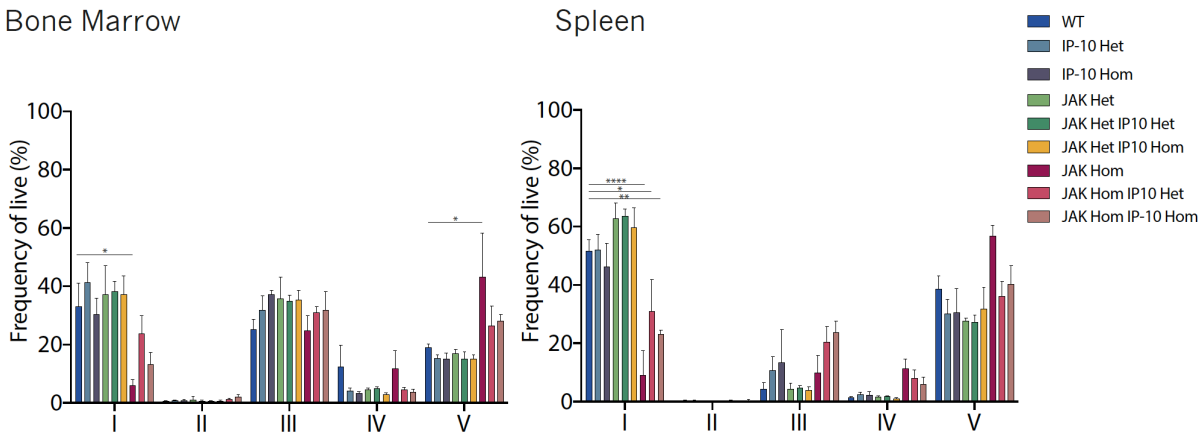


Figure 56. Reduction in CD71^{-}Ter-119^{+} terminally differentiated erythroblasts in JAK-IP-10-mutant mice

BM and spleen cells from JAK- IP-10-mutant mice were isolated and differentiated cell composition analysed (WT, n=2; IP-10 Het, n=4; IP-10 Hom, n=3; JAK Het, n=2; JAK Het IP-10 Het, n=11; JAK Het IP-10 Hom, n=5; JAK Hom, n=2; JAK Hom IP-10 Het n=5; JAK Hom IP-10 Hom, n=3). Representative FACS profiles stained with CD71 and Ter119 antibodies are shown in Figure 11; I through V correspond to progressive stages of erythroid differentiation. Increase of stage V terminally differentiated erythroblast (CD71^{-}Ter119^{+}) is observed in JAK Hom mice compared to WT (p= 0.0487) A slight decrease of stage IV and V erythroblast is observed in JAK Hom IP-10 Het and JAK Hom IP-10 Hom, compared to JAK Hom mice in BM and spleen (stage IV: p=0.9652 and p=0.9668 (respectively) in BM, p=0.8793 and p=0.9990 (respectively) in spleen; stage V: p=0.1582 and p=0.4333 (respectively) in BM; p= 0.0971 and p= 0.4582 (respectively) in spleen). Two-way ANOVA (Tukey's multiple comparisons test).

The earlier results from Section 5.4 showed that JAK TET mutant HSCs maintained the capacity to initiate a PV-like disease when transplanted into an IP-10-lacking environment. Of note, however, loss of IP-10 partially ameliorates the phenotype of JAK- IP-10-mutant mice, as evidenced by a mild decrease in Hb levels alongside a reduction in the proportion of stage V terminally differentiated erythroblast (CD71^{-}Ter119^{+}).

The mechanism of such a phenotypic change would need to be further investigated by extending the phenotyping analysis to a larger cohort of mice and undertaking *in vitro* functional assays (e.g., CFC) on JAK IP-10 cells to demonstrate whether a defect in the erythroid differentiation capacity of JAK-mutant stem and progenitor cells is induced by the loss of IP-10. Finally, it's important to notice that the removal of IP-10 from the disease environment might induce alteration in the levels and/or the production of other cytokines and chemokines, which might be compensating for the loss of IP-10.

Profiling serum samples from IP-10 KO recipients, post-transplantation of JAK TET and JAK HSCs, could help in addressing this concern, and might provide a better understanding of the potential cytokine candidates contributing to the disease phenotype, as well as of the inflammatory changes induced by the loss of the IP-10.

6 Discussion

6.1 Summary of major findings

Emerging studies have highlighted the central role that extrinsic regulators play in modulating HSC fate. A better understanding of the potential players that drive HSC self-renewal, differentiation and/or lineage priming is of direct clinical relevance for the development of gene therapy protocols, for expanding HSCs *ex vivo* for transplantation, and for the production of specific mature cell types[352]. In addition, understanding what factors and downstream pathways are involved in regulating HSC function may elucidate the molecular mechanisms by which the environment regulates HSC fate, as well as the pathophysiological origins of many blood diseases and cancers.

In this thesis, the role of extrinsic regulators of HSCs was explored in the context of both normal and malignant haematopoiesis using the more technically robust mouse HSC system to study the intrinsic regulators of HSC function in a novel hibernation culture system and using primary patient samples to determine the most likely regulators of disease development in pre-leukaemic disorders.

Firstly, in Chapter 3, I describe the cellular and molecular impact of nearly complete absence of cytokine signalling on highly purified single mouse HSCs. By culturing single HSCs in the presence of IL-11 only, it was observed that minimal cytokine stimulation in culture could maintain HSCs in a hibernation state, preventing them from dividing and maintaining their key functional properties *in vitro* and *in vivo* at the single cell level. Although only 20-40% of the initially sorted LT-HSCs survived the absence of cytokines, >99% of surviving cells were maintained as single cells for the entire 7-day culture period and termed hibernating HSCs. Individual hibernating HSCs were still able to generate multi-lineage colonies in CFC assay, and most importantly, were able to reconstitute primary (53%) and secondary (44%) recipients.

Investigation of similarities and differences between freshly isolated HSCs and hibernating HSCs by single cell RNA-sequencing revealed that the two physiological states show a high degree of similarity, with the retention of known self-renewal regulators and more than 80% of cells expressing molecules associated with the G0/G1 phase of the cell cycle. The molecular differences which did exist, however, allowed us to identify key factors potentially dispensable for HSC function. This hibernation culture system was subsequently used to transduce individual undivided LT-HSCs with lentivirus, potentially opening a wide range of possibilities in experimental and clinical research. Finally, the potential translational and technical power of this system was furthered by translating it to human LT-HSCs, with the majority of human cells remaining undivided over the culture period and retaining the capacity to repopulate recipient mice.

Chapter 4 focused on investigating the role of extrinsic regulators in the context of leukaemia development and evolution, using myeloproliferative neoplasms as a model of early-stage tumorigenesis. A number of clinical studies previously showed that some of the constitutional symptoms in MPN patients are concomitant with changes in the serum level of many inflammatory molecules[208,278,285,286]. In addition, MPNs are relatively simple diseases compared to other blood cancers and therefore a useful starting point in which to study the role of such molecules in the context of the HSC clonal advantage required to drive haematological diseases. By profiling the level of different cytokines and chemokines on serum samples from a large cohort of MPN patients, our results identified a strong correlation between certain serum cytokine levels and specific MPN subtypes. In particular, we identified a novel set of inflammatory cytokines that are associated with ET specifically, contrary to previous studies that suggested MF is the only "inflammatory" MPN subtype. Interestingly, we were able to show that levels of 2 of the cytokines analysed (EGF and GRO-α) were associated with disease transformation in longitudinal sampling (EGF) or in diagnostic samples (GRO-α).

Finally, Chapter 5 built from the observation that IP-10 levels in the serum of PV and MF patients was significantly increased and that the highest levels correlated with JAK2 and TET2 mutational status. This implicated IP-10 as a strong candidate

for contributing to the disease phenotype in the most severe MPN subtypes. These findings were complemented by cytokine profiling of MPN mouse models (JAK2, TET2, CALR, and JAK2 TET2), where IP-10 levels also correlated with disease severity and JAK2/TET2 mutations. This raised the possibility of using these disease models to further study the functional impact of the microenvironment on disease establishment and evolution and these data showed that while IP-10 deficiency did not reverse disease phenotype completely, it did lead to a less severe phenotype with respect to haemoglobin levels, terminal erythroid differentiation bias and platelet levels.

6.2 Mouse LT-HSCs can be maintained as fully functional single HSCs in the absence of cytokine stimulation

Studying endogenous regulators of HSCs to resolve the intrinsic heterogeneity existing inside the HSC population has been made possible by advances in single cell RNA-sequencing and the molecular state of freshly isolated HSCs and various progenitor subsets is now well-described in the literature[19,20,50–52]. However, this represents a single physiological state and does not allow any insight into which molecules are central to HSC function versus those that are consequential of being resident in the BM. One possible route around this would be the molecular profiling of *in vitro* expanded HSCs, but current protocols produce an even more heterogeneous population of HSCs and non-HSCs, making the study of self-renewal and differentiation function of HSCs substantially more complicated.

The SCF/KIT and TPO/MPL signalling pathways have been long known to play a crucial role in normal haematopoiesis and have featured in nearly all HSC expansion conditions published to date[26,95,120,131,194,310,334,353]. Changes in SCF concentration *in vitro* have also been shown to modulate HSC self-renewal and differentiation properties at the single cell level[26]. The data in Chapter 3 show that neither SCF nor TPO are absolutely essential to maintain HSC function *in vitro* and the hibernation conditions that are described permit the molecular profiling of highly purified HSCs independent of the BM microenvironment.

The hibernation cultures in Chapter 3 contained IL-11 and previous studies have demonstrated that activation of the gp130 signalling pathway (typically using

IL-6 or IL-11), leads to HSC expansion enhancement *in vitro* and *in vivo*[132,143]. In addition, gp130 is been implicated in different stem cell systems, including Drosophila germ stem cells, with Upd[354], mouse embryonic stem cells with LIF[355], mouse neural stem cells with LIF and CTNF[356], and mouse muscle stem cells with OSM[357]. Importantly, OSM was shown to be able to promote muscle cell engraftment without inducing cell proliferation[357], supporting the hypothesis that gp130 signalling may regulate survival of quiescent stem cells in different systems. The work in this thesis showed that the activation of gfp130 signalling alone, through IL-11 stimulation, was able to maintain LT-HSCs as single multi-potent HSCs during a 7-day culture. These results are in accordance with previous reports showing that inhibition of lipid raft clustering, even in presence of cytokines in the culture media, prevents HSCs from dividing *in vitro* and preserves the ability of HSCs to reconstitute irradiated recipients[135,333]. The same study also showed that this applied to HSCs but not to $CD34^+$ LSK progenitor cells, which all died by 48 hours, suggesting that only primitive cells are resilient to the absence of cytokine signalling.

The finding that single HSCs were able to repopulate primary and secondary recipient mice after the period of hibernation and the patterns of repopulation appeared to be skewed toward the production of myeloid cells (i.e., an α-HSC subtype). Although it would need to be confirmed in a larger number of recipients, the current data suggest that hibernation conditions might preferentially retain α-HSC. This could indicate that the α-HSC subtype is more resilient in the hibernation culture and that the cytokine deprivation might be selecting for the more myeloid-biased HSCs which would also be consistent with their delayed reconstitution kinetics *in vivo*. This hypothesis could be further investigated by isolating purified α-HSCs and comparing to them to β-HSCs, however to date this is not possible based on cell surface marker strategies.

6.3 Hibernation cultures identify molecular regulators potentially dispensable for HSC function

Because their functional properties were almost indistinguishable from freshly isolated HSCs, we used hibernating HSCs as a comparator to identify genes potentially dispensable for HSCs, presuming that those not expressed in hibernating HSCs could

not be essential for HSC function. Gene expression profiling of single LT-HSCs in 7-day hibernation conditions revealed a high retention of self-renewal regulators of the MolO signature, suggesting that such genes are indeed likely to be essential for HSC function and confirming the functional HSC identity of hibernating HSCs at the transcriptional level. A number of regulators were not expressed in hibernating HSCs and their absence did not impact the capacity of cultured cells to successfully engraft primary and secondary recipients. Among those factors were members of the AP1 complex, where expression levels of several protein subunits, including *Jun*, *Fos*, and *Ncor2* were significantly downregulated in hibernation cultures. This could be explained by the fact that proteins of the AP1 complex are normally involved in driving proliferation and therefore their expression is downregulated in hibernating HSCs. This would not be surprising given their role as immediate early gene and stress-activated response genes[358]. Alternatively, the hibernation cultures could potentially be driving the extinguished expression of these factors. If that is the case, it remains formally possible that the expression of these molecules is rescued upon transplantation in order for the cells to proliferate and expand. Re-isolating the hibernating HSCs post-transplantation and studying their transcriptional profile would be interesting in order to assess whether the expression of any of these factors is re-activated when cells are reintroduced into their native niche. That said, it is important to note that SCF-stimulation of the hibernating HSCs (hibHSC+SCF) did not re-initiate their expression, potentially disfavouring this hypothesis.

The molecular profiling also revealed that cell cycle genes were shared between hibHSCs and freshly isolated HSCs, confirming their cell G0/G1 cell cycle state and explaining how their hibernating state might preserve their functional properties. It is reasonable to question whether the hibernation state induced by the reduced cytokine signalling is comparable to the quiescent state in which HSCs are found when residing in their physiological environment and it is difficult to formally prove this in the absence of a robust set of quiescence characteristics. That said, there are some assays that could be performed to further investigate the cellular state of cells including Hoechst/Pyronin staining or single cell immunofluorescence for key cell cycle regulators. These *in vitro* assays are quite challenging to perform at the single cell levels and/or on low number of cells in culture.

Interestingly, among the genes upregulated in hibernating HSCs, we identified a number of pro-survival genes including *Ier3, Pdcd1lg2*, and HLF targets, such as *Lyz2* and *Lrcc8a*. The HLF pathway has been previously implicated in HSC biology and recently proved to be a critical regulator of HSC quiescence[344], further supporting the hypothesis that the hibernation period could resemble a quiescent-like state *in vitro*. Of additional interest is that HLF itself seems to associate with HSCs in all cellular states to date[359], suggesting it may also be a component that primes HSCs to re-enter quiescence, but further work would need to be performed to investigate this hypothesis.

6.4 Hibernation conditions as a tool to genetically manipulate single LT-HSCs

The knowledge that HSCs are fully functional during the hibernation cultures offers the opportunity to genetically manipulate them at the single-cell level without the traditional worry of HSC differentiation with longer culture periods. The data in this thesis show that lentiviral constructs can be delivered in hibernation cultures, which could set the stage for the delivery of multiple viral constructs to introduce multiple genetic modifications in single functional HSCs during a relatively short culture period. This could also allow the study of combinatorial genetic modification in a pool of more highly purified HSCs, compared to current methods which transduce a heterogeneous population of stem and progenitor cells typically obtained in *ex vivo* culture protocols.

Further working toward this goal, our data also show that hibernation conditions might be translatable to human HSCs, since a substantial fraction can be maintained as single cells for 7 days in minimal cytokine conditions. This would offer the attractive clinical and experimental potential to assess the possibility of genetically modifying human HSCs and to optimise cytokine combinations which could lead to further enriching for repopulating human HSCs.

6.5 Inflammatory factors in myeloproliferative neoplasms

The work described in Chapters 4 and 5 leveraged a large and comprehensive patient dataset with complete genetic information to identify and subsequently characterise the functional role of specific candidates in the disease evolution of MPNs. The

investigation of extrinsic regulators in MPNs candidate molecules for having a distinct role in the disease biology of different MPN subtypes. For example, IP-10 and TNF-α levels were frequently increased in MPNs, with significant patterns seen in patients of the more severe subtype (MF) compared to the other MPN subtypes. While the association between myelofibrosis and a highly inflammatory disease environment has been long known and described, our data also revealed an unexpected presence of specific pro-inflammatory cytokines in less severe disease (GRO-α, Eotaxin and EGF).

These results raise the question of whether the observed up-regulation of specific cytokines is directly involved in disease progression or whether they are simply a by-product of cytokine-secreting cell types. Most importantly, it remains unclear whether the differences in cytokine levels are due to secretion by cells arising from the mutant clone or whether their production is triggered indirectly by the effects of the tumour clone on the non-tumour cells. Therefore, cytokine screens such as this should be complemented by profiling the mature cell fractions of patient samples to determine the cellular source and its origin (or not) within the mutant clone driving the malignancy. Such studies would help establish if more cytokines are produced on a per-cell basis or just as a result of higher cell number. However, obtaining the genotype and the cell surface profile of the same cells still remains a challenge, and is further compounded by comparisons between patients with differently sized mutant clones. Once the cell producing the differentially expressed cytokines is established, it would also be crucial to determine which cells or tissues are the targets of such molecules and whether these are main players in supporting the disease.

6.6 The potential role of IP-10 in MPN disease pathogenesis

Multiple studies have implicated interferon signalling in both HSC function[85,87,360] and MPN biology[271,349,361]. Interferon gamma-induced protein-10 (also known as CXCL10) is a chemokine secreted in response to IFN-γ. IP-10 binds the cell surface chemokine receptor CXCR3 that is present on most peripheral blood memory T cells and at higher levels on Th1 cells. It is produced by a number of different cells, including monocytes, lymphocytes, keratinocytes, and endothelial cells. Importantly, IP-10 has been identified as advantageous factor for tumour growth in a number of cancers where

higher expression of IP-10 and/or its receptor CXCR3 (CD183) seems to correlate with poor prognosis[362–364].

The MPN cytokine profiling in this thesis showed a strong correlation of IP-10 levels with increasing disease severity, with elevated levels associated with the worst disease subtypes in patients as well as with the JAK2 TET2 mutational status in both patients and mouse models, suggesting a potential involvement in driving disease severity. The similarity between patient and mouse data highlight the value of these models, not only re-capitulating the human disease, but also showing similar trend of cytokine levels comparable to the patient screen. In fact, as observed in MPN patients, IP-10 is similarly dysregulated in these models: its levels are increased in JAK and TET models, with an even greater increase in JAK TET double mutant models. This suggests that a number of similarities might be present between the human and the mouse microenvironment and confirm these models as excellent tool to characterise the role and impact of IP-10 in MPN pathogenesis.

Our *in vitro* data on human and mouse HSC culture show no effect on HSC proliferation and survival. In addition, expression of the IP-10 receptor (CXCR3) on HSCs has not been reported before, suggesting that HSCs might not be directly affected by an IP-10-enriched environment *in vitro* and that IP-10 might be acting on different cell targets. Alternatively, as previously reported for other cytokines, the effect of IP-10 on HSCs might be different *in vivo* but this hypothesis has not been verified. Further investigation of potential targets of IP-10 will be required to clarify the exact effect (if present at all) of this cytokine on the environment. Importantly, verifying whether the over-production of IP-10 is due to the mutant or WT clone overproducing it in the disease microenvironment could help identify potential strategies to target the IP-10 producing cells.

The transplantation of JAK TET mutant HSCs into IP-10 KO background showed that disease could still be initiated in the transplantation setting. Importantly, the donor graft contains cells that might eventually secrete IP-10, re-introducing the cytokine into the recipient environment or additional cytokines may be upregulated in response to the absence of IP-10 to fulfil the same role as previously described for

numerous other cytokines[365]. Screening the serum of these recipient mice could help establish whether IP-10 has been reintroduced and/or whether other cytokines have been produced in higher levels to compensate the lack of IP-10 (and possibly their function). That could then help identify other contributors and further investigate the effect of depleting IP-10 on the other inflammatory factors secreted in the BM environment and one could also imagine targeting multiple molecules simultaneously.

The JAK2V617F KI/IP-10 KO (JAK IP-10) cross we generated to address this concern also develops a PV-like phenotype comparable to the JAK mutant mice, although some mild reductions were observed in red cell and platelet production. All that said, the increase in IP-10 levels in mouse models appears to be even more starkly driven by the TET2 mutation, and a JAK2V617F KI/TET2 KO/IP-1 KO cross might be able to investigate whether the mutant clone expansion is dependent on the presence of IP-10 in the cytokine environment or the increase is meanly a reaction of the immune system to the disease. To investigate the clinical importance of IP-10, it would also be interesting to treat JAK TET mice with an IP-10 blocking antibody, to study whether reduction of IP-10 levels in serum could affect the mutant clone expansion and improve the disease phenotype.

6.7 Exploring the clinical importance of cytokine profiling

Since the discovery of specific gene mutations in MPNs[222–227], the field has rapidly progressed with advanced treatment and diagnosis options. Available treatments include anti-inflammatory agents such as steroids, aspirin or immuno-modulatory agents[228,231,235,294], which are mainly directed at decreasing the number of certain mature blood cell types and reducing the risk of complications. Over time, traditional chemotherapies are being replaced by targeted immunotherapy in the treatment of solid tumours[366], and several antibody-based therapy options are already available[367]. In CML, the discovery of the BCR-ABL mutation represented an immeasurable advance in targeted therapy, as it allowed the development of efficient protein-specific drugs (e.g. Imatinib[368]). Our data identified a number of inflammatory cytokines that are elevated in chronic phase MPNs. Previous reports have shown that ET patients have relatively low levels of cytokines and chemokines compared to PV

and MF patients[204]. However, these studies often included very few patients (n=5 in Ho[293] et al, n=21 in Pourcelot et al[286], n=15 in Boissinot et al[208]). Given the huge heterogeneity existing across patients with the same disease, screening a large number of patients was important to identify potential significant changes in cytokine levels.

Genetic mutations in myeloid malignancies have been increasingly used for predicting disease outcome[328,369]. The choice of the type of treatment depends on the MPN subtype and/or the severity of the disease with no single treatment being effective for all MPN patients. On the other hand, subtype diagnosis is relatively inconsistent and recent molecular profiling data suggests that substantial heterogeneity can be resolved by including driver mutation profiling in disease classification/prognosis. However, investigating extrinsic regulators have resulted in an increased interest since it has become obvious that genetic mutations cannot fully explain disease heterogeneity. This could also be due to different stages of disease evolution[370], different sized mutant clones[371], different orders of mutation acquisition[238], or other factors not yet tracked in these models. The work in this thesis demonstrated that cytokine profiling has prognostic value for predicting transformation, in addition to age, sex, and comprehensive genomics. High levels of GRO-α were predictive of MF transformation in both the initial and follow-up cohorts and decreasing EGF levels over time could predict for transformation in longitudinal sampling. Longitudinal measurement in EGF levels also reaches beyond the binary assessment of mutant/non-mutant and allele burden that is often done with genetic profiling and can give a dynamic measurement of changes in disease evolution (more similar to minimal residual disease monitoring in lymphoid malignancies and CML).

Despite the powerful potential clinical implication of such a discovery, further work is required to establish the mechanisms and the role of these cytokines. Several outstanding questions remain: 1) what is driving the differences in cytokine levels? 2) Are these differences due to the secretion by cells arising from the mutant clone or is their production triggered indirectly by the effects of the tumour clone upon non tumour-cells?

Clearly, the number of variables to take in account to evaluate disease pathogenesis extends beyond the genetic mutations harboured by each individual. Integrating biological, genetic and clinical dataset could help define which inflammatory factors play a central role in the disease and how they contribute to its transformation while also informing decision making in the clinic. The possibility of identifying and monitoring individual inflammatory factors that can drive clonal evolution might improve the current diagnostic and treatment practice and reduce the risk of transformations into more severe disease or even to AML and reduce their impact on tumour development.

6.8 Conclusions

Overall, this PhD thesis highlighted the importance of extrinsic factors as critical regulators of HSC function as well as potential contributors to the pathogenesis of pre-leukaemic diseases. It showed that removal of SCF and TPO cytokine signalling can maintain HSCs as single cells *in vitro* for extended period of time, while preserving their *in vitro* and *in vivo* stem cell properties, indicating that such signalling is not essential for the maintenance of HSC function. Importantly, keeping HSCs in this state of hibernation permitted the study of their molecular properties and facilitated the identification of factors dispensable for their function while also providing a new platform to genetically manipulate them while retaining full functional properties.

These findings open new possibilities for *in vitro* manipulation of highly purified HSCs and may have substantial implications for the culture and expansion of human HSCs aimed at autologous stem cell transplantation and for potential pre-treatment applications in allogenic stem cell transplantation. Since MPNs are genetically less complicated than numerous cancers, they represent a highly tractable system for studying the cytokine microenvironment and to characterise its role in the establishment and progression of haematological malignancies. The findings from these studies could therefore be taken forward and tested for their applicability to more severe or less accessible cancers, in particular for diseases with such short life expectancy as AML. Perhaps most surprisingly, this thesis reveals the power of using cytokine profiling for disease monitoring and identified a set of cytokines differentially expressed in MPNs and potential biomarkers of disease severity and/or

evolution. This work highlights the importance of the inflammatory factors in the context of the disease and will set the stage for further investigation into their functional role on HSCs, as well as for further consideration in the pathogenesis of MPNs and other cancers.

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