Characterisation of Immune Cell Subsets in Mouse Models of Myeloproliferative Neoplasms

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Abstract

Recent studies have implicated the role that the microenvironment plays in disease evolution. Changes in specific molecules can directly modulate haematopoietic stem cells (HSCs) to make different numbers and types of cells and this suggests that the microenvironment might mediate disease development. My thesis explores this in the context of myeloproliferative neoplasms, a set of clonal HSC-derived disorders that are considered pre-leukaemic. Specifically, I will assess the impact of CXCL10 (or IP-10) on HSPCs and mature cell production in an allelic series of models.

Studies have previously shown that notably higher levels of CXCL10 are associated with increased disease severity in both human patient studies and mouse models of MPNs. This has been demonstrated by serum cytokine profiling and correlation to disease severity in both patients and mice. In my thesis, I, therefore, explored the impact of CXCL10 loss on disease severity, using flow cytometry in mouse models with either JAK2 V617F mutations, TET2 mutations, or CXCL10 mutations (or combinations of these), Mice deficient in IP-10 show slower MPN disease development with reduced erythrocytosis. This work established that CXCL10 impacted the numbers and types of cells in the bone marrow. Next, I went on to validate genes expressed in these cell subsets by performing qRT-PCR, identifying a key regulator of disease.

Together, my findings highlight the importance of extrinsic cellular regulators and support the theory that changes in the haematopoietic microenvironment can alter cellular fate outcomes in the malignant setting, my work highlights the need to undertake future investigations to identify cellular and molecular pathways that might be targeted to improve patient outcomes.

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Abbreviations

7AAD; 7-Aminoactinomycin D
5hmC; 5-methylcytosine
5mC; 5-hydroxymethylcytosine
ANOVA; Analysis of variance
AML; Acute Myeloid Leukaemia
APC; Allophycocyanin
ARCH; Age-related clonal haematopoiesis
Aslx1; Additional sex combs like 1
Bcl-xL; B-cell lymphoma-extra large
BM; Bone marrow
Bmi1; B lymphoma Mo-MLV insertion region 1 homolog
BV; Brilliant violet
CALR; Calreticulin
C/EBPα; CCAAT/enhancer-binding protein alpha
cDNA; Complementary deoxyribonucleic acid
CLP; Common lymphoid progenitor
CML; Chronic myeloid leukaemia
CMP; Common myeloid progenitor
CAR; Chimeric antigen receptor
CXCL12; Chemokine motif ligand 12
CXCR3; Chemokine motif receptor 3
DMSO; Dimethyl sulfoxide
Dnmt3a; DNA methyltransferase 3 alpha
EGF; Epidermal growth factor
ER; Endoplasmic reticulum
ET; Essential thrombocythemia
Ezh2; Enhancer of zeste homolog 2
FACS; Fluorescence-activated cell Sorting
FCS; Foetal calf serum
G-CSF; Granulocyte colony-stimulating factor
GMP; Granulocyte-monocyte progenitor
GRO-α; CXCL1
Hb; Haemoglobin
HSC; Hematopoietic stem cell
HSPC; Hematopoietic stem and progenitor cell
Idh1/2; Isocitrate dehydrogenase 1/2
IFN; Interferon
IFN-γ; Interferon-gamma
IL; Interleukin
IP-10/CXCL10; Interferon gamma-induced protein 10
JAK2; Janus Kinase 2
KI; Knock-in
KO; Knock-out
Lin; Lineage
LSK; $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
NF-κB; Nuclear factor-kappa B
MCL1; Myeloid cell leukemia 1
MEP; Megakaryocyte-erythroid progenitor
Meis1; Myeloid ecotropic viral integration site 1
MF; Myelofibrosis
MPL; Myeloproliferative leukemia virus oncogene
MPN; Myeloproliferative neoplasm
MPP; Multipotent progenitor
MSC; Mesenchymal stromal cell
PB; Peripheral blood
PBS; Phosphate buffered saline
Pbx1; PBX homeobox 1
Ph; Philadelphia chromosome
PIM1; Pim-1 proto-oncogene
pMF; Primary myelofibrosis
PV; Polycythaemia vera

qPCR; Quantitative polymerase chain reaction
RBC; Red blood cell
SCF; Stem cell factor
SDF1; Stromal cell-derived factor 1
SLAM; Signalling lymphocyte activation molecule
SEM; Standard error of the mean
Sca-1; Stem cell antigen-1
STAT; Signal transducers and activators of transcription
TET2; Ten-Eleven Translocation 2
TNFα; Tumour necrosis factor-alpha
TPO; Thrombopoietin
TGF-beta; Transforming growth factor beta
UPR; Unfolded protein response
WT; Wild type

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Authors Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references.

1.0 Introduction

1.1 Haematopoiesis & the haematopoietic hierarchy

Haematopoiesis is a highly regenerative process responsible for the daily production of approximately 1 trillion (10^{12}) mature blood cells, taking place within the adult bone marrow (BM). Haematopoiesis is arranged into a hierarchal system which maintains and regulates the formation and replenishment of the blood system (1). The blood is responsible for a wide variety of essential bodily functions including the transport of oxygen and nutrients to tissues and the fighting of diseases (the innate and adaptive immune systems). At the apex of the haematopoietic hierarchy exists a rare population of cells, haematopoietic stem cells (HSCs), which have the unique ability to differentiate into the different mature cell progeny necessary to complete the normal function of the mammalian blood system (2). This process is a hierarchical one, where HSCs give rise to progenitor cell intermediates, which are highly proliferative, and these eventually lead to the functional, mature components of the blood system.

Broadly speaking, the blood system is divided into two branches: Erythro-myeloid and lymphoid cells. Erythro-myeloid cells include red cells, platelets, macrophages, and granulocytes (basophils, neutrophils & eosinophils). Erythrocytes, or red cells, contain haemoglobin which binds O_2 and travels from the lungs to tissues and organs. Platelets, which are fragments of megakaryocytes play an important role in blood clotting and wound repair. Macrophages are highly versatile cells involved in the phagocytosis of pathogens, dead cells and debris; they relate to the adaptive immune system through their antigen presentation ability to T-cells. Granulocytes carry nutrients and are involved in innate immunity. The lymphoid cells come in two main types that can be categorised as innate lymphoid cells (e.g., natural killer (NK) cells) which kill cells without any priming (e.g., the clearing of virally infected cells) and adaptive immune cells such as B and T cells. While B cells are responsible for producing antibodies, T cells are responsible for killing cells and mounting an adaptive immune response.

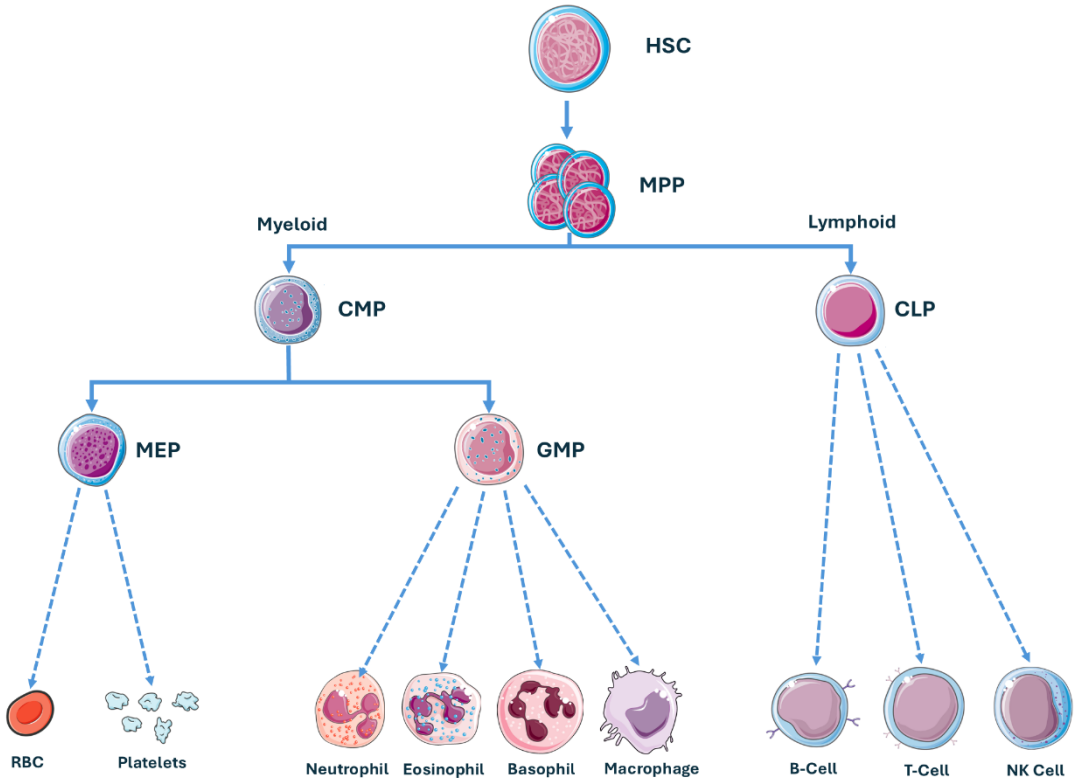

Figure 1 – Graphical representation of haematopoietic hierarchy and cells involved. Haematopoietic stem cells (HSC) reside at the apex of the hierarchy and differentiate into multi-potent progenitors (MPP), which commit to the myeloid or lymphoid lineages. The myeloid lineage gives rise to common myeloid progenitors (CMP), which can differentiate into megakaryocyte-erythroid progenitors (MEPs) and granulocyte-monocyte progenitors (GMPs). MEPs ultimately form mature erythrocytes and platelets, while GMPs give rise to neutrophils, eosinophils, basophils, and macrophages. The lymphoid lineage produces common lymphoid progenitor (CLP), differentiating into B cells, T cells, and NK cells. This figure was generated using Sevier Medical Art, provided by Servier, licenced under a CC-BY 3.0 unported licence.

Ensuring that the supply of these cells is regulated and sufficient, HSCs and their downstream progeny divide and differentiate in a coordinated fashion and this process is regulated by haematopoietic cytokines and transcription factor activation (reviewed in (3-7)). These factors are provided largely by other cell types surrounding the stem and progenitor cells in the bone marrow, where the vast majority of HSCs reside. HSCs are thought to first transition to multipotent progenitors (MPPs) and then

split into more restricted progenitor cell types as they proceed down the differentiation cascade. (Figure 1)

MPP cells commit to myeloid and lymphoid lineages, differentiating into common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), respectively. CMPs give rise to cells of the myeloid lineage, further differentiating into committed granulocyte-macrophage progenitors (GMP), which develop into granulocytes (neutrophils, eosinophils, and basophils) and megakaryocyte-monocyte progenitors (MEP) which develop into megakaryocytes and erythrocytes (8). GMP differentiation is regulated by various growth factors and transcription factors which guide the development of the myeloid lineage during normal haematopoiesis (reviewed in (9)). CLPs give rise to cells of the lymphoid lineage, including T-cells, B-cells and, NK cells (10)(Figure 1).

Overall, this balance is critical for providing the correct numbers and types of blood cells on a daily basis and when this balance is disturbed, disease can arise.

1.2 The stem cell niche

Within the bone marrow, there exists a highly regulated microenvironment, or niche, that controls the quiescence, proliferation, and differentiation of HSCs (11). During homeostasis, most HSCs remain quiescent (12) but can become activated through regulatory signals produced by niche-inhabited cells, in response to intrinsic or extrinsic challenges (13). Interferon-mediated signalling can initiate the proliferation and differentiation of HSCs within the niche. For example, TNF-α, a type I IFN that causes increased HSC proliferation via the activation of Signal transducers and activators of transcription 1 (STAT1) and PBL/Akt pathways (14).

The niche is comprised of multiple key cell types which support the maintenance of HSCs (**Figure 2**). The niche is highly vascular, containing a number of perivascular cells which influence cell fate. Endothelial cells within the stem cell niche are essential to establishing and maintaining the vasculature and have been shown by several groups to be involved in driving HSC decision-making (15-17). Two essential molecular factors which support HSCs in the niche are CXCL12 (and its cognate receptor SDF1) and SCF (with receptor c-Kit) (15, 18). Perivascular mesenchymal stromal cells (MSCs), specifically Leptin receptor-expressing (LepR^{+}) MSCs are major sources of key factors such as chemokine (C-X-C motif) ligand 12 (CXCL12) and stem cell factor (SCF) (15, 18), which are essential regulators of HSC maintenance retention within the bone marrow. (15, 19, 20).

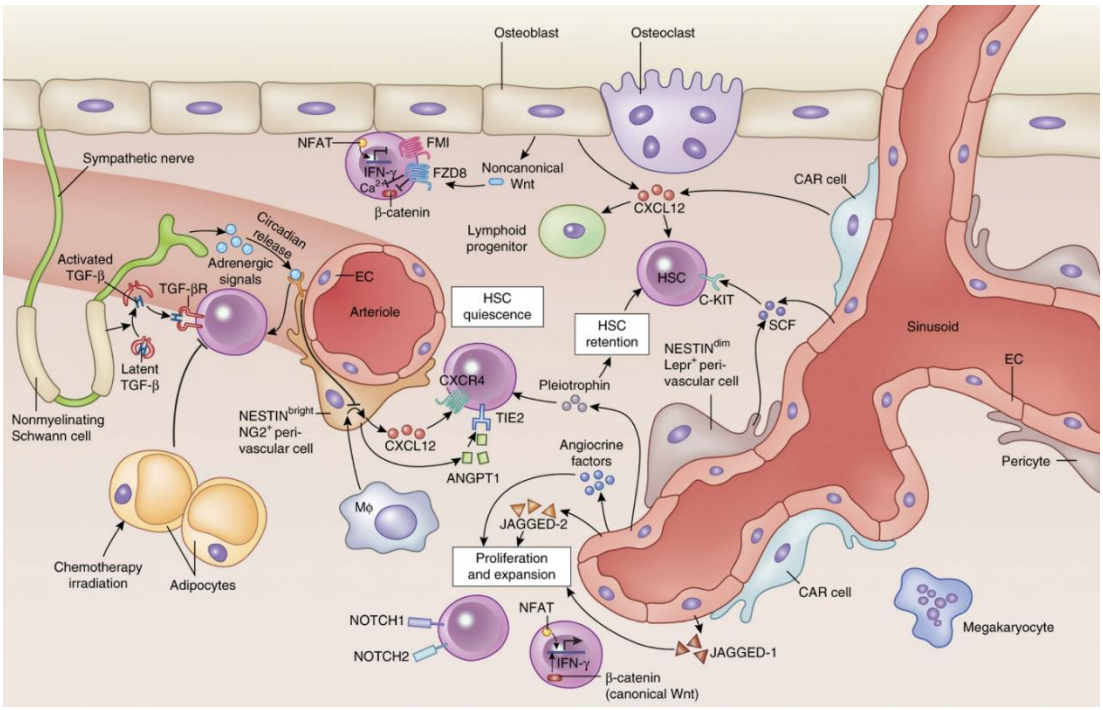

Figure 2 - Cell types implicated in promoting HSC maintenance. Including endothelial cells (ECs), CAR cells, macrophages, perivasascular stromal cells, sympathetic neurons and nonmyelinating Schwann cells. They are shown to interact with haematopoetic stem cells (HSC) through expression of regulatory factors such as CXCL12 and SCF. Reproduced from (*Mendelson, et al.* 2014) with permission from Springer Nature.

1.3 Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPNs) are a unique classification of clonal haematopoietic stem cell disorders, collectively linked by mutations which result in constitutively active signal-transduction pathways involved in haematopoiesis, resulting in dysregulated production and function of mature myeloid cells. They result in the overproduction of mature cell types and about 10% of patients progress to more severe diseases, including leukaemias. The prototypical MPN is chronic myeloid leukaemia (CML) which has benefitted from the identification of the unique BCR-ABL fusion gene that results in a chromosomal translocation that in turn results in the formation of a new chromosome named the Philadelphia chromosome (Ph).

Ph-negative (Ph^-) MPNs, the subject of this thesis, can be broadly categorised into three distinct disorders: polycythaemia vera (PV), essential thrombocythaemia (ET), and myelofibrosis (MF). PV and ET are considered to be milder forms of disease but can progress to more severe disease and require regular monitoring.

Ph^- MPNs are a heterogeneous group of clonal HSC malignancies, characterised by somatic mutations affecting primarily three driver genes: *JAK2*, Calreticulin (*CALR*) & *MPL*, all of which encode signalling proteins responsible for directly or indirectly inducing upregulation of the Janus-kinase (JAK)-signal transducer and activator of transcription (STAT) pathway in MPNs (21, 22). Frequent co-mutations in MPNs are somatic mutations in epigenetic regulators of DNA methylation, in particular, the Ten-Eleven Translocation-2 (*TET2*) gene, is commonly co-mutated in JAK2 V617F MPNs in ~10% of human patients (23).

1.4 JAK2V617F mutation

JAK2^{V617F} is the most common phenotypic driver mutation associated with all three of the MPN subtypes: Polycythaemia vera (PV), Essential thrombocythemia (ET) and myelofibrosis (MF) mutated in all of the main Ph$^-$ MPN subtypes. This mutation is by far the most abundant and is present in ~95% of PV and ~60% of ET and PMF cases (24). The V617F mutation drives hyperactive JAK-STAT signalling activating transcription of genes **(Figure 3)** involved in cell proliferation, survival and apoptotic resistance (eg. Bcl-xL, Cyclin D1, MCL1, and PIM1) (25-27)

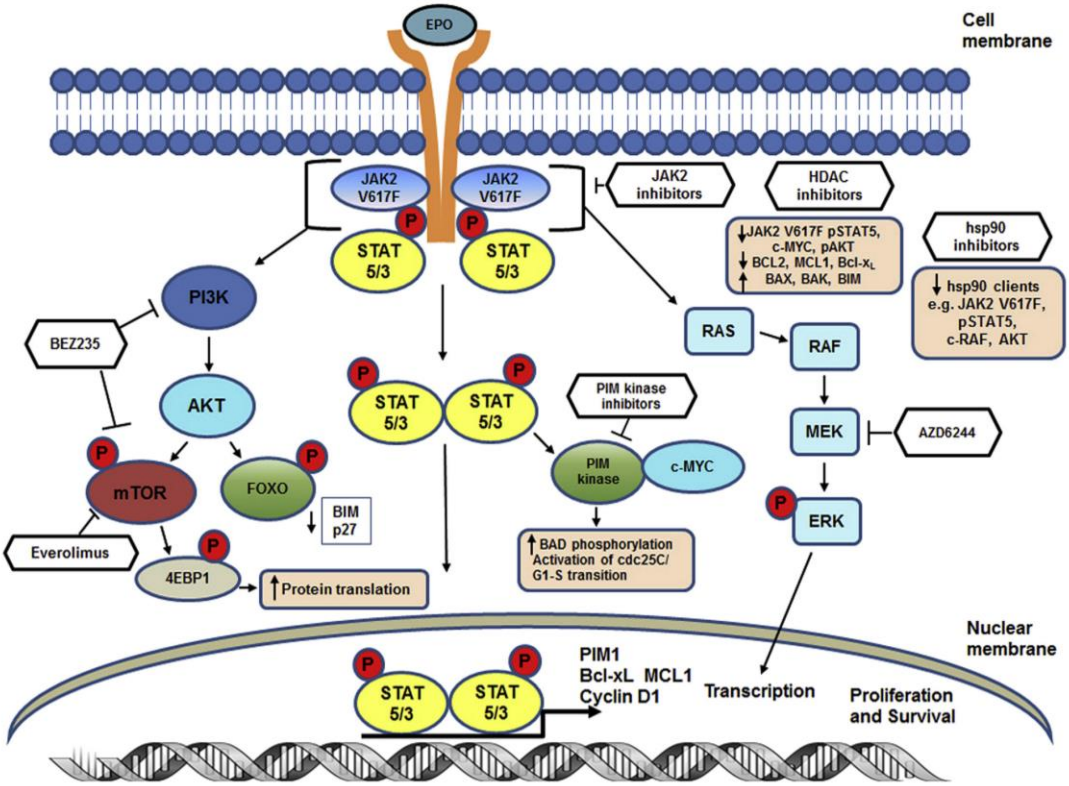

Figure 3 – Overview of JAK V617F signalling pathways. Reproduced from (*Fiskus et al.* 2012) with permission from Elsevier.

The other two main driver mutations are *CALR* or *MPL* mutants, and these are currently thought to be restricted to MPL activation.

1.5 Calreticulin mutations

Calreticulin (*CALR*) is a major endoplasmic reticulum (ER) chaperone involved in several cellular functions, including Ca^{2+} homeostasis and protein folding. *CALR* has been implicated in the development of MPNs such as ET and pMF (Primary myelofibrosis) (28).

In 2013, two groups published reports implicating the Calreticulin (*CALR*) gene in MPNs (28, 29). They discovered *CALR* mutations affected 60-80% of patients with JAK2 and MPL negative MPNs - particularly ET and pMF. MPN patients who are $JAK2^{V617F}$-negative (29), display similar activation of the JAK-STAT signalling pathway with distinct clinical and haematological features compared to $JAK2^{V617F+}$ patients with increased platelet levels and lower thrombotic risk (28, 29).

CALR mutations are detected at the HSPC level, consistent with a role in initiating both ET and pMF diseases. Most *CALR* mutations are classified as type 1 or type 2, categorised by 52 bp deletions (*CALR* del52) and 5 bp insertions (*CALR* ins5) (30, 31). Type 1 mutations are typically associated with a MF phenotype with higher risk of fibrotic transformation towards ET (30), while type 2 mutations are more common in ET (31). Both mutant variants, *CALR* del52 and *CALR* ins5 contribute to MPN pathogenesis by impairing critical cellular responses to the unfolded protein response (UPR) and oxidative stress management, increasing cellular damage and apoptosis, further stressing HSCs and progressing disease (32).

1.6 Other mutations in MPNs

There are a wide range of other mutations that have been implicated in MPN including those in other cytokine signalling pathways (e.g., MPL) and a large number of epigenetic regulators such as Asxl1, Idh1/2, Dnmt3a, Ezh2, etc. These are all catalogued in a number of recent exome and genome studies involving MPN patient

samples but will not be discussed further in this thesis, with the exception of TET2 mutations, an epigenetic modifier that is the subject of this thesis.

1.7 - TET2 mutations

The Ten-eleven translocation 2 (*TET2)* gene encodes one of the three proteins within the TET family of enzymes, TET enzymes are capable of altering the epigenetic status of DNA though the methylation and conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) (33-35). Somatic loss of TET2 function through monoallelic or biallelic loss-of-function mutations and deletions have been frequently observed in MPNs (35, 36). Such mutations reduce TET2 activity, resulting in aberrant DNA methylation matters and disrupted gene regulation which is discussed later.

1.8 Mouse models of MPNs

Since the discovery of the JAK2 V617F mutation in 2005, a number of groups have undertaken mouse modelling of MPNs (37-41). The first set of these were retroviral overexpression models which showed a marked erythrocytosis (42), these were followed by sophisticated transgenic mice with different copy numbers of the mutant allele (43, 44), and finally, 4 groups made knock-in mouse models where the JAK2 V617F mutation was under the control of the endogenous JAK2 promoter and enhancer elements (45-48). The various characteristics of these mice are summarised below in Figure 4.

Broadly speaking, JAK2^{V617F} mice typically develop an MPN-like phenotype with amplification of mature myeloid cells and LSK/SLAM populations resulting in disease progression towards a myelofibrosis-like phenotype. In the model used in this thesis, homozygous JAK2 V617F mice give rise to a PV-like phenotype with splenomegaly and large numbers of erythroid cells (49).

Shepherd *et al.* showed that while JAK2^{V617F} mutant HSCs have a high proliferative advantage, they lack sufficient self-renewal capabilities to initiate disease (50). However, compound TET2 mutations exacerbate the phenotype when JAK2^{V617F} mice were crossed with *TET2* KO mice, malignant HSCs had both the hyperproliferative advantage in addition to increased self-renewal, providing a clonal advantage to JAK2^{V617F} HSCs through increased expression of key self-renewal regulators (*Bmi1, Meis1, and Pbx1)* (51).

Dufour et al. created an IP-10 deficient KO mouse model, which established the role of IP-10 in effector T-cell generation and trafficking (52). Following several key studies, IP-10 was directly implicated in MPN disease progression and phenotype severity (53, 54). *Belmonte et al.* crossed the IP-10 KO mice with an allelic series of JAK2 KI and TET2 KO mice and demonstrated that loss of IP-10 significantly dampened the exacerbated erythrocytosis associated with JAK2 and TET2 MPN pathogenesis (55). These combinatorial IP-10 KO mice will be the focus of this thesis.

Table 1. Retroviral overexpression, transplantation and transgenic mouse model phenotypes

A. Retroviral overexpression and bone marrow transplantation models

V617F species	Recipient	Hct (%)		Platelets	WBC	MF	Reference
Mouse	BALB/c	73.7±6.5 (VF)	49.7±4.3 (WT)	~2-fold increase	~17-fold increase	Yes	Wernig et al., 2006
	C57Bl/6	72.5±3.8 (VF)	44.9±2.5 (WT)	Normal	~2-fold increase	No	
Mouse	C57Bl/6	61±7 (Group 1; VF) 57±7 (Group 2; VF)	Values not shown	Normal	Increased	Yes	Lacout et al., 2006
Mouse	BALB/c	~70 (mean; VF)	~40 (mean; WT)	Normal	Increased	Yes	Zaleskas et al., 2006
	C57Bl/6	~65 (mean; VF)	~47 (mean; WT)	Normal	Mild increase	Yes	
Mouse	BALB/c	~83 (median; VF)	–	Normal	Increased	Yes	Bumm et al., 2006

B. Transgenic models

V617F species	Construct	Activation	Hct (%)	Hb (g/l)	Platelets (×10⁹/l)	WBC (×10⁹/l)	MF	Reference
Mouse	*H2Kb* promoter	Not inducible	49.9±2.9[a] 42±4.5[b]	>180; 20% mice[a] 100 (mean)[b]	>1400; 35% mice[a] 2500 (mean)[b]	>20; 35% mice[a] 30 (mean)[b]	Yes NA	Shide et al., 2008
Human	*Vav* promoter	Not inducible	50.9±4.42[c] 48±2.87[d]	181±14[c] 165±5.9[d]	2708±712[c] 1278±215[d]	11.8±2.85[c] 9.14±2.63[d]	Yes No	Xing et al., 2008
Human	Minimal human *JAK2* promoter	*Vav*-Cre	47±1.67	164±5.93	3710±488.2	13.06±3.73	Yes	Tiedt et al., 2008
		Mx1-Cre:						
		pIpC ×6	49.33±9.87	161.3±32.33	8883±4461.7	28.63±21.08	Yes	
		pIpC ×3	51.09±6.35	182.2±19.71	2792±1267.2	14.12±8.00	Yes	
		pIpC ×1	61.60±9.10	196.0±42.07	5316±4315.6	35.62±35.57	Yes	

Table 2. Summary of JAK2V617F knock-in mouse models

Ref.	V617F species	Activation	Mouse background	Allele expression and validation	Animal survival	Het. V617F phenotype	Hom. V617F phenotype	Spleen
Akada et al., 2010	Mouse	pIpC induced	129Sv/C57Bl/6 mix	50% of WT expression; constitutive phosphorylation (Stat5, Erk, Akt)	Some death, unknown cause	PV-like (Hct ~80%; Plt ~1.5×10^{12}/l; WBC ~2- to 3-fold increase)	PV-like (Hct ~80%; Plt ~3.5×10^{12}/l; WBC, ~5- to 7-fold increase)	Enlarged
Mullally et al., 2010	Mouse	Constitutive	129Sv/C57Bl/6 mix	Expression lower than WT	Median survival = 146 days	PV-like (Hct ~80%; Plt, no significant change; WBC ~2-fold increase)	NA	Enlarged
Marty et al., 2010	Mouse	Constitutive	129Sv/C57Bl/6 mix	Expression similar to WT; constitutive phosphorylation (Stat5, Erk)	5 of 11 died (15±3 weeks)	PV-like (Hct ~70%; Plt ~4×10^{12}/l; WBC ~7.3-fold increase)	NA	Enlarged
Li et al., 2010	Human	pIpC induced	129Sv/C57Bl/6 mix	Expression similar to WT; no constitutive pStat5; hypersensitive to Epo	Normal unless transformed	ET-like (Hct ~60%; Plt ~1.6×10^{12}/l; WBC, mild increase; 10% mice transformed to PV or MF)	NA	Not enlarged

Table 2. Continued

Ref.	MF	Disease transplantability[a]	Effect on lineages	Colony formation	Effect on stem/progenitor cells	HSC function
Akada et al., 2010	Mild in het. BM; high in hom. BM; high in het. spleen; high in hom. spleen	Yes (D, backcrossed 4G; R, C57Bl6)	10-fold increased $Ter119^+CD71^+$ (20-fold in homozygous); 6- to 10-fold increased $Mac1^+Gr1^+$ (only observed in spleen)	Increased: GM (spleen); BFU-e (spleen and BM); GEMM (spleen); EECs (spleen)	2-fold increase LSK (BM and spleen); increased MEPs	NA
Mullally et al., 2010	None in BM or spleen (up to 6 months)	Yes (D, unknown number of backcrosses; R, C57Bl6)	2-fold increased $Ter119^+CD71^+$ (BM); 3-fold increased $Ter119^+CD71^+$ (spleen); no change in $Mac1^+Gr1^+$	Increased: BFU-e; EECs absent	No change LSK; increased $L^-S^-K^+$ (more MEP and pre-CFU-e)	No difference in competitive BMT (B6 recipients)
Marty et al., 2010	BM and spleen (~30 weeks)	NA	88-fold increase in erythroid precursors; 83-fold increase in myeloid precursors	Increased: G, M, BFU-e, CFU-e; EECs present	NA	NA
Li et al., 2010	None in BM or spleen (up to 6 months)	Yes (D, backcrossed 2G; R, 129SvEvBrd/C57Bl6 F1)	Mild increase in $Ter119^+CD71^+$; mild increase in $Mac1^+Gr1^+$ (only observed in BM)	Increased: G, M, BFU-e; EECs present	Reduced LSK in aged mice (normal in young)	Reduced function in competitive and non-competitive transplantations (129/B6 F1 recipients)

Figure 4 – Overview of retroviral, transplantation and transgenic mouse models and the characteristics of the resulting MPN phenotypes in mice. Reproduced from (*Li et al.* 2011) licenced under a CC BY-NC-SA 3.0 unported licence

1.9 The MPN microenvironment

One of the puzzling features of MPNs is how patients with the same genetic mutations have such distinct clinical phenotypes. One of the potential factors influencing this is the immune cell microenvironment and the involvement of an inflammatory microenvironment in myeloproliferative neoplasms has been described in multiple studies (53, 54, 56-58). Overproduction of multiple pro-inflammatory cytokines has been observed in MPN patients, including tumour necrosis factor-alpha (TNF-α) (57).

Malignant cells and their niche interact dynamically, altering the cellular microenvironment and reinforcing disease development through a positive feedback loop (59, 60). (**Figure 5**). Inflammatory signalling is increased in MPNs, this elevated inflammatory cytokine expression creates a permissive environment for malignant clonal expansion (61, 62). Increased inflammation preceding the development of MPN disease creates a permissive microenvironment, enabling the expansion and survival of the mutant clone, contributing to disease evolution (63). Previous research has suggested that chronic inflammation is a hallmark of MPNs, and the progression of malignant HSCs are directly linked to changes within their niche (21).

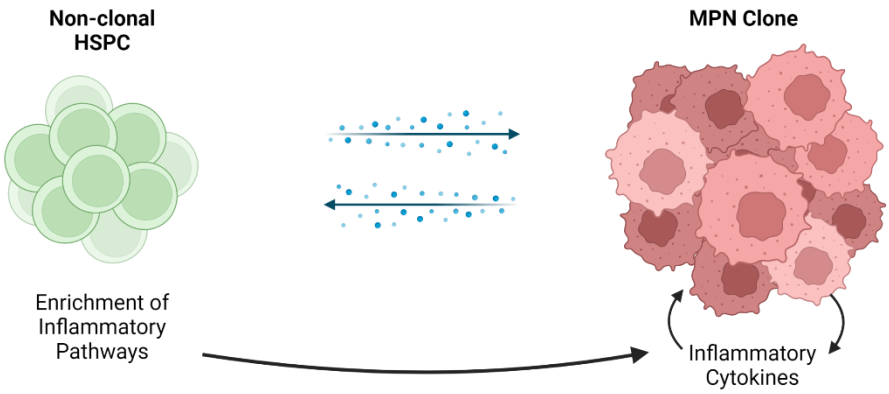

Figure 5 - Involvement of inflammatory cytokines in promoting clonal dominance of mutant haematopoietic stem and progenitor cells. Created in BioRender.

One of the less studied molecules that is also increased is the chemokine interferon gamma-induced protein 10 (IP-10 or CXCL10) (64). IP-10 is a chemokine

secreted in response to upregulation of IFN-γ (65). IP-10 binds to CXC chemokine receptor-3 (CXCR3), which is typically expressed in activated T-cells allowing IP-10 to induce, specifically through CXCR3 a wide variety of potent physiological effects in cell growth, and development such as tumour regulation, promotion and angiogenesis (65). IP-10 is a key pro-inflammatory cytokine which is highly expressed in mutant but not wild-type JAK2 MPN patients and is associated with increased disease severity when present with additional *TET2* co-mutations (54). Tefferi *et al.* have suggested that elevated levels of IP-10 in human MPN patients is an independent marker of poor prognosis. The expression of IP-10 is thought to be directly induced by downstream Nuclear Factor-κB (NF-κB) activation through constitutive $JAK2^{V617F}$ signalling (66).

In 2020, *Obro et al.*, undertook a cytokine screen in patients with complete genetic characterisation to determine whether patients had distinct microenvironments. They found that patients with ET had significantly elevated levels of inflammatory cytokines (GRO-α and EGF) (53). In addition to these findings, a follow-up study implicated IP-10 as a potential modulator of MPN evolution (55).

Interestingly, the Belmonte study found that mouse models of JAK2 V617F, CALR, and TET2 (and mutation combinations) also showed this pattern of increased levels of serum IP-10 in mice with the most severe phenotype (**Figure 6**), making mice an excellent model for studying the immune cell populations and their perturbations in the context of MPN pathogenesis.

Understanding the complexity of the stem cell microenvironment during disease development is a complex task but doing so in less complex diseases such as MPNs has many advantages. Moreover, the fact the genetic models exist for the key intrinsic regulators (JAK2 and TET2 mutations) made this disease highly tractable for assessing the role of a microenvironmental factor such as IP-10.

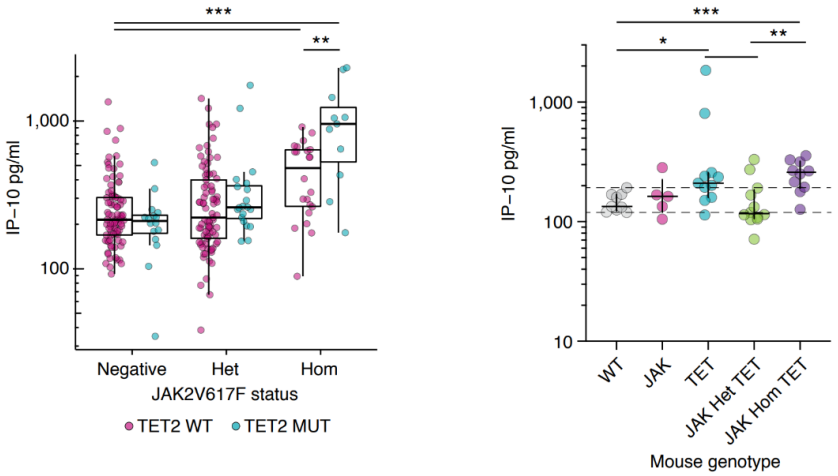

Figure 6 – Serum levels of IP-10 correlate with JAK2 and TET2 mutational status. IP-10 serum levels in patients with JAK2 and TET2 mutations. IP-10 levels in mouse models of myeloproliferative neoplasms. Reproduced from (*Belmonte et al.* 2024) licenced under a CC-BY 4.0 licence.

Thesis Aims

Utilising established mouse models of MPNs (JAK2, JAK2/TET2), and with the introduction of IP-10 KO models (JAK2/IP-10, JAK2/IP-10/TET2) deficient in IP-10, this thesis aims to assess the role of IP-10 loss in disease pathogenesis. This will be investigated through flow cytometric analysis of cellular subsets involved in MPN progression, alongside gene expression analysis of C/EBP**α,** a gene critical for regulating myeloid differentiation at a progenitor level. The aim is to explore whether IP-10 deficiency affects differentiation at the HSPC level or influences terminal differentiation of mature cell subsets in the context of MPNs. Investigating IP-10 is of particular interest as studies have shown is a potential biomarker implicated in the development and severity of MPNs.

This thesis therefore aims to:

1) Characterise the stem and progenitor cell subsets of MPN mouse models
2) Characterise the mature cell composition of MPN mouse models
3) Understand the gene expression of C/EBPα in stem and progenitor cell subsets

2.0 Materials and Methods

2.1 Mouse Models

This project utilised multiple mouse models of myeloproliferative neoplasms, with differing disease characteristics. Original models include a *JAK2V617F* knock-in (KI) (50), an IP-10 knock-out (KO) (67) and a TET2 KO (35). For this thesis, $JAK2^{V617F}$ KI, $JAK2^{V617F}$ KI / IP-10 KO) and triple-mutant mice with $JAK2^{V617F}$ KI, IP-10 KO & TET2 KO were used.

A combination of frozen murine whole bone marrow and fresh mouse bone marrow harvested from 8–12-week-old mice was used throughout this thesis. Due to a limited number of mature mice available frozen whole bone marrow encompasses most of our samples. All mice were bred and maintained at the University of York in microisolator cages and provided continuously with sterile food, water, and bedding. All mice were kept in specified pathogen-free conditions, and all procedures were performed according to the United Kingdom Home Office regulations under Project Licence PEAD116C1.

2.2 Freezing cells

Whole bone marrow collected from mice was frozen in a 20% DMSO solution in foetal calf serum (FCS) (freezing medium) and stored in liquid nitrogen. Cells were prepared in 1mL FCS and transferred into two cryovials, each containing 500uL of prepared cells. 500uL of 2X freezing medium was then added drop-by-drop until the final volume was reached before being vortexed and placed on ice. Cells were moved to a Mr. Frosty™ Freezing Container and moved to -20^{o}C for 24 hours to maintain an optimal cooling rate for cell preservation. Cells were then moved to -80^{o}C and stored until use.

2.3 Red blood cell lysis

Red blood cell lysis was performed on fresh mouse bone marrow cells using a 10% solution of ammonium chloride. Cells were centrifuged at 300 x g for 5 minutes, before resuspending in 3mL of PBS 2% FCS. 5mL of cold ammonium chloride was added and incubated on ice for 5 minutes, followed by 15 seconds of vortexing before being incubated on ice for another 5 minutes. Following incubation, the solution was diluted with 12mL PBS 2% FCS and centrifuged at 300 x g for another 5 minutes. The supernatant was removed, and the lysed cells were resuspended in 100uL PBS 2% FCS/mouse and transferred to a 5mL polystyrene tube for staining and lineage depletion.

2.4 Lineage depletion & antibody staining

As a de-bulking step, lineage depletion was performed using the EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit (Stemcell Technologies, Catalog #19856) as per the manufacturer's protocol but with only 1 enrichment step. Following depletion, the EasySep™ Mouse Hematopoietic Progenitor Isolation Cocktail was added along with the rest of the antibody staining panel at a dilution of 1:200 to allow discrimination to true lineage-negative cells. Collectively, lineage-positive cells (CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119, 7-4) were removed during FACS when tagged with fluorescent streptavidin (BV510). Only samples where mature cells were not the target population were lineage-depleted to avoid skewing of percentages or removal of key cell types.

Two antibody panels were used during this project, one to characterise the mature cell compartments of the mouse models (CD45, B220, CD3e, CD11b, Ly-6G) without lineage depletion (Table 1) and the other to characterise and sort progenitor & HSC populations (CD16/32, CD150, CD48, CD117, CD34, Ly-6A/E, CD135) following lineage depletion (Table 2).

2.5 Antibody panels

Mature Cell Compartment

Marker	Fluorochrome	Clone	Cat. No.	Concentration
CD45	Alexa Fluor 700	30-F11	BioLegend 103128	1:200
CD45R/B220	APC	RA3-6B2	BioLegend 103212	1:400
CD3e	PE	145-2C11	BioLegend 100308	1:200
Mac1 (CD11b)	PE-Cy7	M1/70	BioLegend 101212	1:200
Ly-6G	BV421	1A8	BioLegend 127628	1:200

Table 1 – Antibody staining panel used to identify mature cell populations via flow cytometry.

HSPC Compartment

Marker	Fluorochrome	Clone	Cat. No.	Concentration
CD16/32	PE	93	BioLegend 101307	1:100
CD150 (SLAM)	PE-Cy7	TC15-12F12.2	BioLegend 115914	1:100
CD48	APC	HM48-1	BioLegend 103412	1:100

CD117 (c-Kit)	APC-Cy7	2B8	BioLegend 105811	1:100
CD34	FITC	RAM34	BD Pharmigen 553733	1:100
Ly-6A/E (Sca-1)	BV605	D7	BioLegend 108133	1:100
CD135 (FL2K)	BV421	A2F10	BioLegend 135315	1:100
Streptavidin	BV510	N/A	BioLegend 405233	1:200
EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Cocktail	Biotinylated Antibodies against: CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119		StemCell 19856C	1:200

Table 2 – Antibody staining panel used to identify haematopoietic stem and progenitor cell populations following lineage depletion via flow cytometry.

2.6 Isolation of progenitor populations from whole mouse bone marrow

Both fresh and frozen samples were used to sort the progenitor populations (GMPs, CMPs, MEPs) and LT-HSCs (LSK/SLAM population) from whole mouse bone marrow. Using the CytoFLEX SRT Benchtop Cell Sorter & BD FACSDiscover™ S8 Cell Sorter located at the Biosciences Technology Facility at the University of York. GMPs, CMPs & MEPs contained within the myeloid progenitor population ($Lin^{-}c\text{-}Kit^{+}$ (LK)) were characterised by expression of CD16/32 and CD34, SLAM populations were defined by $CD48^{-}CD150^{-}$. Representative plots shown in Figure 7 and panels in Table 3.

Progenitor population	Marker Phenotype
Granulocyte-monocyte progenitor (GMP)	$Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD34+CD16/32^{+}$
Common myeloid progenitor (CMP)	$Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD34^{+}CD16/32^{-}$
Megakaryocyte-erythrocyte progenitor (MEP)	$Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD34^{-}CD16/32^{-}$

Table 3 – Surface markers to isolate and sort haematopoietic stem and progenitor populations via fluorescence activated cell sorting (FACS).

2.7 RNA extraction

Various cell populations were collected into 1mL RNAse free Eppendorf's containing 500uL PBS 10% FCS before proceeding with RNA isolation using the Applied Biosystems™ Arcturus™ PicoPure™ RNA Isolation Kit (Cat: KIT0204). RNA isolation and extraction was performed on the sorted populations by centrifuging the cells immediately after sorting at 5000 x g for 5 minutes, supernatant was carefully removed and 50uL of Arcturus Picopure Extraction Buffer (XB) was added and gently resuspended before being incubated at 42^{o}C for 30 minutes on a benchtop dry heating block. Following incubation cells were centrifuged at 3000 x g for 2 minutes and the supernatant containing the extracted RNA was placed into a new 0.5mL DNA LoBind® Eppendorf for storage until further processing.

2.8 RNA isolation

The RNA purification columns provided in the kit were preconditioned with 250uL Conditioning Buffer and incubated at room temperature for 5 minutes before centrifugation at 16,000 x g for 1 minute. A matching 50uL volume of 70% Picopure EtOH was added to the RNA cell extract and pipetted briefly. The cell extract & EtOH

mixture was then added to the preconditioned purification column and to bind RNA to the column, centrifuged at 100 x g for 2 minutes followed immediately by 16,000 x g for 30 seconds to remove flowthrough. 100uL of the provided Wash Buffer 1 (W1) was added to the column and centrifuged again at 8000 x g for 1 minute. All samples then underwent a DNase treatment using the RNase-free DNase Set from Qiagen (Cat. No: 79254). A DNase incubation mix was created using 5uL DNaseI stock solution diluted in 35uL Buffer RDD DNA Digest Buffer. 40uL of DNase incubation mix was added to each purification column membrane and incubated for 15 minutes at room temperature. 40uL of W1 was then added into the purification column membrane and centrifuged at 8000 x g for 30 seconds.

2.9 cDNA Synthesis

Isolated RNA was converted into cDNA for use in quantitative polymerase chain reaction (qPCR) using ThermoFisher SuperScript™ IV Reverse Transcriptase. 50uM Oligo$(dT)_{20}$ Primer was added into a reaction mix containing 10mM deoxyribonucleotide triphosphate (dNTP) & template RNA before being annealed at 65°C for 5 minutes. To convert the annealed RNA into cDNA a reverse transcriptase mix (RT) containing: 5x SuperScript IV Buffer, 100mM DTT, RNaseOUT Recombinant RNase Inhibitor and SuperScript IV Reverse Transcriptase (200U/ul) was added and heated to 52.5°C for 10 minutes then inactivated at 80°C for 10 minutes.
To reduce nonspecific amplification of PCR targets, 1uL of *E. coli* RNase H was added and incubated at 37°C for 20 minutes.

2.10 qPCR

1uL of diluted cDNA (100ng/uL) was mixed with 5uL TaqMan™ Universal PCR Master Mix (2X), 0.5uL TaqMan™ Primer Mix before plating. Plates were analysed on an Applied Biosystems™ QuantStudio™ 7 Flex located at the Biosciences Technology Facility at the University of York. The TaqMan Comparative Ct Standard run template was used.

2.11 Statistics and Data Analysis

Quantitative data were analysed using GraphPad Prism 10 software and Microsoft Excel. Unless otherwise stated, an ANOVA was used to compare individual pairs or groups of samples respectively. Unless otherwise stated, the p values are represented by *$p<0.05$; **$p<0.01$ and ***$p<0.001$

3.0 Results

The results in this thesis focused on exploring the impact on blood and immune cell subsets of adding an IP-10 knockout onto the JAK2/TET2 double mutant background. Investigations focused on whether the removal of IP-10, which has been implicated by previous work as a driver of disease severity, would alter the stem and progenitor cell or mature cell frequency in mouse models.

To achieve this, the Kent lab first undertook a series of mouse breeding to create combinations of mutations involved in disease pathogenesis of myeloproliferative neoplasms and crossed IP-10 KO into those mice to assess whether or not it was essential. I then surveyed the resulting animals by flow cytometry for changes in the stem and progenitor cell fractions and the mature cell fractions. Following this survey of cell types, I then moved toward undertaking quantitative RT-PCR to assess gene expression in these cellular subsets. This work aims to determine how the loss of IP-10 affects HSPC populations, as well as mature cell subsets, and whether this loss has an impact on gene expression at the transcriptional level, focusing on C/EBPα.

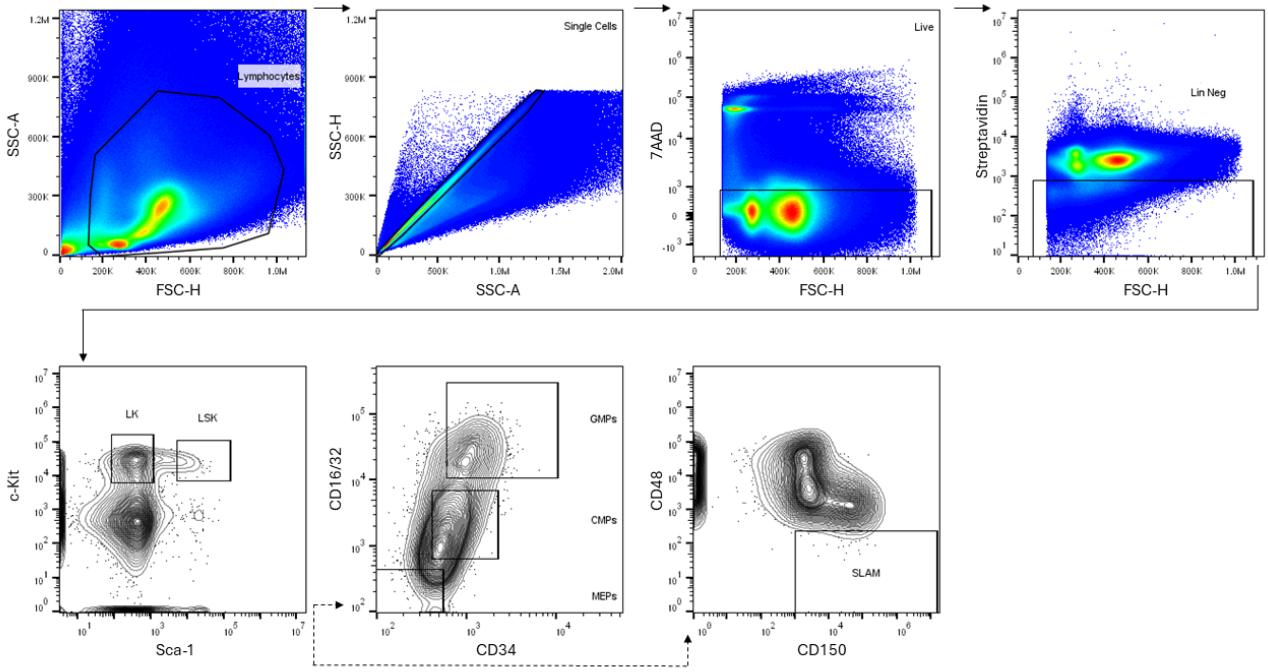

Figure 7 - Representative gating strategy for isolating progenitor populations from murine whole bone marrow as analysed by flow cytometry. *Lin neg; Lineage Negative, LK; Lin$^-$c-Kit$^+$, LSK; Lin$^-$c-Kit$^+$Sca-1$^+$, GMPs; Granulocyte-monocyte progenitors, CMPs; Common myeloid progenitors, MEPs; Megakaryocyte-erythroid progenitors, SLAM; Signalling lymphocyte activation molecules.*

3.1 - Immune cell phenotype of JAK2 mice

JAK2 mutant mice have been previously described as having a strong red cell phenotype reminiscent of PV in humans (42). Peripheral blood count data shows an increased haematocrit and increased haemoglobin alongside mild splenomegaly and occasional transformation to more severe MPNs or spontaneous cardiothoracic events (68). Here we first asked the question of whether mature cell types were different and found that there was a statistically different change in granulocytes between J and J/I mice in murine whole BM (Figure 8d).

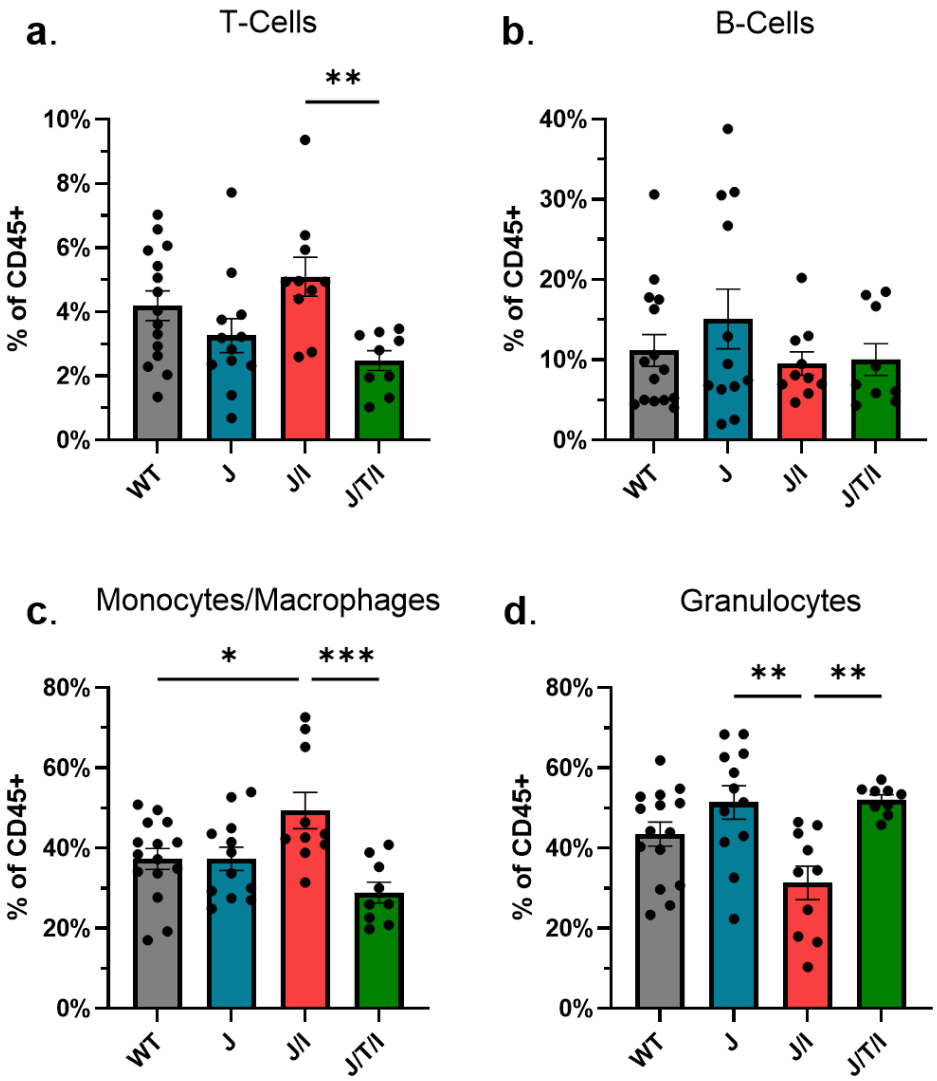

Figure 8 - Flow cytometry analysis of murine whole bone marrow shows significant differences in mature myeloid populations between mouse models of MPNs. (a) T-cells,

(b) B-cells, (c) Monocytes/Macrophages, and (d) Granulocytes were analysed as a percentage of total $CD45^+$ cells. Data is shown as mean ± standard error mean (SEM), with each dot representing an individual mouse. Statistical analysis was performed using one-way ANOVA for **a**, **b**, and **d**. were normally distributed following Shapiro-Wilk tests, followed by post hoc multiple comparisons. For **b**., where data did not pass Shapiro-Wilks normality tests, a non-parametric Kruskal-Wallis test was applied with post hoc multiple comparisons. Significant differences are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. WT; Wild type, J; JAK, J/I; JAK/IP-10, J/T/I; JAK/TET/IP-10.

Next, I assessed whether the stem and progenitor cell compartments were changed. Whereas HSCs have been previously reported to be variably reduced in JAK2 V617F homozygous mice, results show significantly reduced numbers in both CMP **(Figure 9a)** and GMP **(Figure 9b)** fractions alongside a slight reduction in MEP fractions compared to the other mutants **(Figure 9c).** This is largely consistent with the phenotype of increased production of red cells that is observed in the gross morphology of the mouse model and the stem cell defect observed in transplanted animals. In short, the V617F mutation pushes the HSCs to divide more frequently and when cells transition to the progenitor stage, they are more likely to make red cell and platelet progenitors (MEPs) which subsequently give rise to the mature cells driving the phenotype.

3.2 - Immune cell phenotype of JAK2/IP-10 mice

JAK2/IP-10 mutant mice have been previously described as having a striking erythroid cell over-production which involved higher haemoglobin and haematocrit as well as increased spleen sizes. This was initially puzzling as we suspected that IP-10 loss would dampen the erythrocytosis. Largely the JAK2/IP-10 double mutant mice phenocopied the JAK2 mouse as seen in **Figure 8 and 9**.

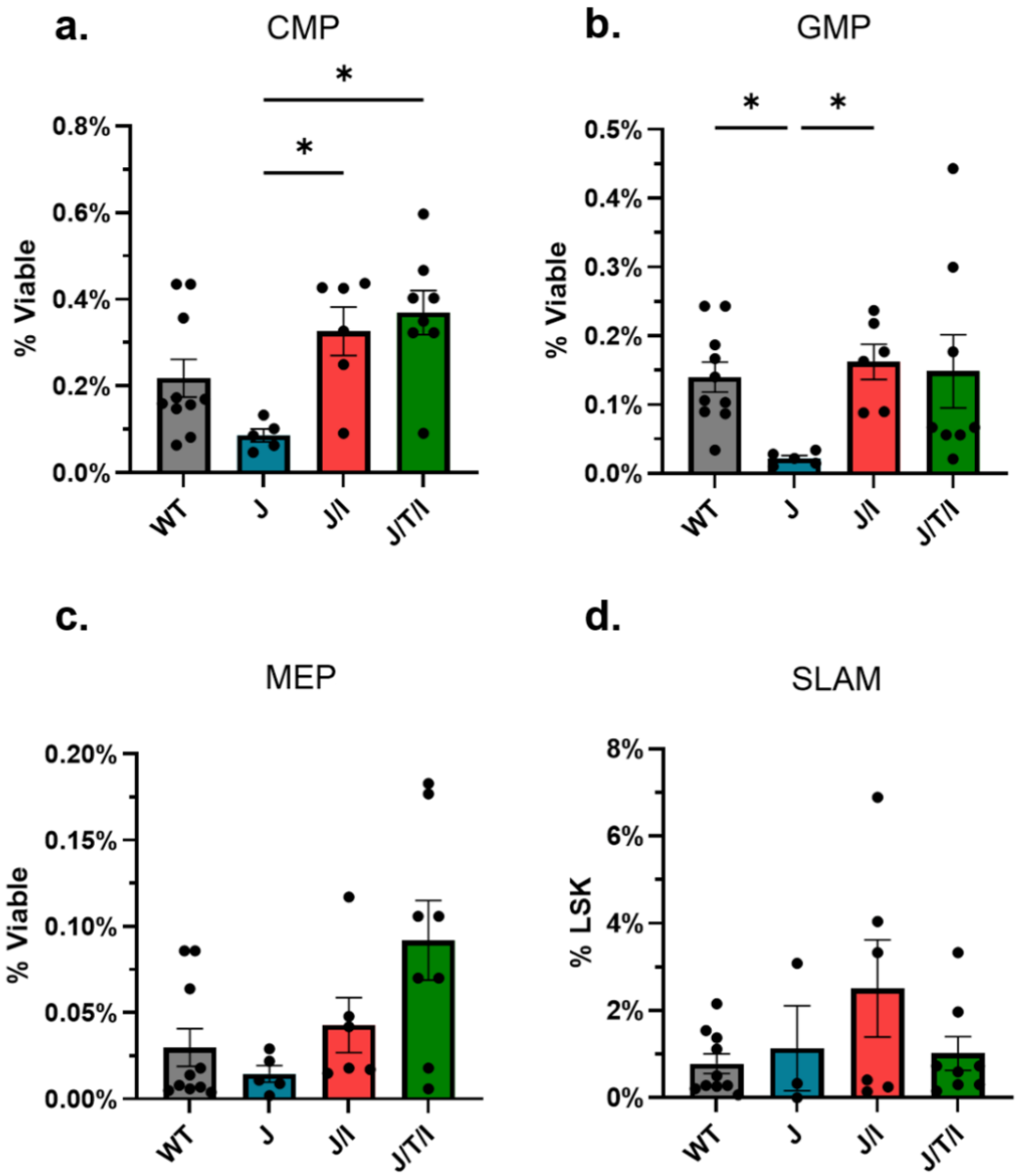

Figure 9 – Increased CMP and GMP populations between mouse models of MPNs.

a. Common myeloid progenitors (CMP), b. Granulocyte-macrophage progenitors (GMP), c. Megakaryocyte-erythroid progenitors (MEP), and d. SLAM populations were analysed as a percentage of total viable cells. Data show significant increases to the CMP and GMP compartments in J/I and J/T/I mice compared to WT and J, while MEP and SLAM populations showed no significant differences. Data is shown as mean ± SEM, with each dot representing an individual mouse. Statistical analysis was performed using the non-parametric Kruskal-Wallis test followed by post hoc multiple comparisons due to data being non-normally distributed. Significant differences are indicated as follows: *p<0.05. WT; Wild type, J; JAK, J/I; JAK/IP-10, J/T/I; JAK/TET/IP-10.

3.3 Immune cell phenotype of JAK2/IP-10/TET2 mice

When I assessed the TET2 triple mutant mouse, I observed that several cell subsets appeared to resolve and moved closer to the wild type. Specifically, T-cells (**Figure 8a),** monocytes/macrophages (**Figure 8c**), granulocytes **(Figure 8d)** and GMP subsets (**Figure 8b).** Suggesting that IP-10 loss in the context of TET2 mutant MPNs has a broader impact on these populations compared to IP-10 loss alone.

3.4 qPCR analysis of CMPs

Next, I assessed the expression of C/EBPα in murine CMPs to determine whether it was altered in these cells. I also attempted to assess the expression of C/EBPα in GMPs, MEPs and SLAM cells through qPCR, however, these experiments were unsuccessful due to technical issues, and the data was not included in this thesis.

CMPs represent a critical branch point in myeloid progenitor commitment. By quantifying C/EBPα expression - a key transcription factor crucial for regulating granulopoiesis and balancing the transition of CMPs into GMPs & MEPs (69-71). I explored whether IP-10 loss influences differentiation bias at the CMP level. Such bias could skew lineage commitment of CMPs towards GMPs or MEPs which is often observed in MPNs. Data were log-transformed the 2^{-}**(ΔΔCt) va**lues and a non-parametric Kruskal-Wallis test was run to determine statistical significance (**Figure 10**).

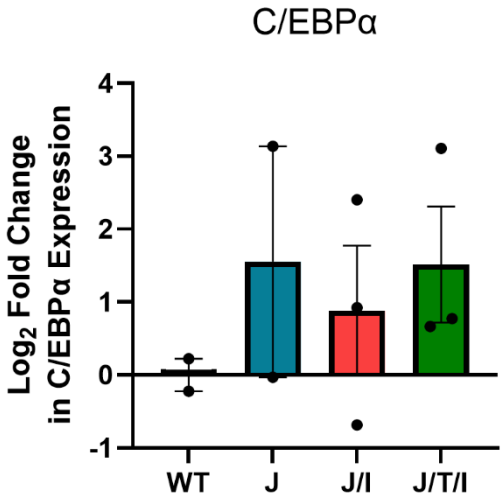

Figure 10 – qPCR of C/EBP-α in common myeloid progenitor cells. Individual dots are individual mouse samples and data are analysed as described in methods.

Figure 10 displays the log_2 fold change in C/EBP-α expression across mouse models. Each dot represents an individual data point, highlighting the variation in gene expression within each group. Given the small sample size (WT; n = 2, JAK; n = 2, JAK/IP-10; n = 3, JAK/TET/IP-10; n = 3), variability within samples is more pronounced, which can affect the interpretation of the results, and more data would be required for making firmer conclusions.

That said, the overall trend appears to indicate upregulation of C/EBP-α in J, J/I and J/T/I mice although not statistically significant (Kruskal-Wallis test, p = 0.6392, post hoc Duns test shows p-value = >0.999 between each sample). This was more pronounced in J and J/T/I but with such variability, it is difficult to determine without additional samples which would be the subject of future studies. Future studies with larger sample sizes and genes of interest would be useful to strengthen our findings, as they could reduce uncertainty and provide clearer conclusions.

4.0 Discussion

4.1 Summary of major findings

Numerous studies have demonstrated the importance of the cellular microenvironment in regulating normal haematopoiesis and regulating the development of disease. A greater understanding of the molecules that drive cancer progression is of direct clinical relevance for the development of new therapies that target the drivers of cancer evolution. In addition, deciphering which pathways are involved in regulating MPNs specifically may allow us to pinpoint the molecular mechanisms by which the environment regulates malignant HSCs and give clues as to the origins of blood cancers in general.

In this thesis, I explored the role of IP-10 in driving the extrinsic regulation of stem and progenitor cells as well as mature cells in a triple mutant mouse model of MPNs. IP-10 was identified as a major player in disease biology, but no studies have ever genetically modified it in the context of MPNs. I took advantage of the existence of a triple mutant mouse model and profiled immune cell subsets and stem/progenitor cell subsets to determine whether this molecule, which strongly correlates with disease severity in patients, influenced these cell populations.

In my results chapter I show the flow cytometry data for single and double mutant mice as well as data from the triple mutant which overall pointed to a number of mature and immature cell types as being disrupted in each of single, double and triple mutant mouse models. The loss of IP-10 resulted in shifts in some of these cell subsets. I followed this up with gene expression profiling of C/EBPα, a key transcription factor involved in progenitor differentiation and observed a trend of upregulation across mutant mouse models compared to wild-type, while this increase was not statistically significant. This trend could reflect intrinsic or extrinsic mechanisms influencing C/EBPα expression, compensating for the already

dysregulated differentiation and inflammation profiles typically observed in MPNs. It would be interesting for further studies to utilise a wider selection of genes and cell targets to gain a wider understanding of the roles the inflammatory microenvironment has on both HSPC differentiation and mature cell lineage commitment.

Our models open up the possibility to be used to further study the functional impact of the microenvironment on disease establishment and evolution and potentially targeting IP-10 to restore haemoglobin levels, terminal erythroid differentiation bias and platelet levels would be of use in mice and eventually in patients.

4.2 Molecular profiling of cells

In my thesis, I provided flow cytometry and qPCR data, but these are both reliant on knowing the target prior to the experiment. In future, it would be highly interesting to undertake global gene expression and proteomic profiling of cell populations from the triple mutant mice (and all of the controls) to be able to then undertake gene set enrichment analyses, pathway analyses and to potentially discover new players in the pathogenesis of MPNs. Another possible route in cases where cell numbers are prohibitive for the technology being proposed, we could grow the cells in the new mouse HSC expansion conditions (72), where mutated and non-mutated cells could be compared both to obtain large numbers and also to study any differentiation biases.

4.3 Inflammatory microenvironment in myeloproliferative neoplasms

The work described in my results utilised combinatorial mouse models of MPNs, which have highly similar relative levels of cytokines that track with genotype. This implies that the genetic changes are also driving microenvironment changes and that these changes could be derived from the mutant immune cells which exist for decades in patients prior to disease development. This suggests that a potentially long period of time for HSCs and progenitors being exposed to additional

inflammatory strain can lead to HSC exhaustion, leading to a permissive microenvironment that supports malignant transformation and survival of mutant driver clones, directly contributing to MPN disease initiation and evolution (63). This could be one of the key reasons why age matters for MPN development. Multiple studies have implicated interferon signalling in both HSC function and MPN biology (45, 73, 74). Interferon signalling has also been identified as a driver of tumour growth in several cancers with IP-10 levels correlating with poor prognosis. In normal organisms, IP-10 is secreted by a number of different cells, including monocytes, lymphocytes, keratinocytes and endothelial cells, in response to IFN-γ.

5.0 Conclusions

Overall, this master's thesis highlights the importance of mouse models which combine the same mutations in patients into the models and also look for indirect changes that are mirrored (e.g., genetic changes lead to similar environmental changes). This type of interactivity across the systems to produce similar inflammatory strain in combination with the development and amelioration of phenotype observed (55), is compelling evidence that these models are accurate. My findings open new possibilities for flow profiling and gene expression studies, but perhaps more importantly, they point to a need to undertake further multi-omic studies to fully understand the impact of IP-10 on MPN development and pathogenesis. The 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 and progenitors, while also implicating new pathways in the pathogenesis of MPNs and other cancers.

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