Understanding instructive cytokine signalling in preleukaemic haematopoietic stem cells

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Abstract

Haematopoietic stem cells (HSCs) differentiate into mature blood cells through the control of multiple cytokines at various branch points and HSCs are capable of adjusting haematopoiesis in response to different physiological triggers. In many patients with a range of myeloid malignancies, loss of function TET2 mutations have frequently been observed. TET2 has been found to be a critical regulator of self-renewal and differentiation of HSCs and many of the responses of HSCs to cytokines have been shown to be impacted by TET2 mutations. We sought to study the cytokine response of wildtype and TET2 mutated HSCs to gain insight into whether specific pathways might contribute to explaining the differing cell fates between mutant and non-mutant HSCs. We found that $TET2^{+/-}$ HSCs are hyporesponsive to IL-4 and exhibit slower cell cycle kinetics in response to IL-4, IFN-γ, TGF-β and IL-6. We also demonstrated that IL-6 and TGF-β promote differentiation of $TET2^{+/-}$ HSCs and $TET2^{+/-}$ HSCs produce fewer granulocytes in response to TGF-β. In addition, our data showed that $TET2^{+/-}$ HSCs do not demonstrate reduced colony forming efficiency in response to IL-4 and IFN-γ. It was also noticed, through this project, that $TET2^{+/-}$ untreated cultures have elevated Sca-1 expression which highlights the emergence of a hyperinflammatory state in myeloid malignancies with TET2 mutations such as AML. Further research will be necessary to explain the reasons why many of these differential cell fate decisions occur.

Graphical abstract

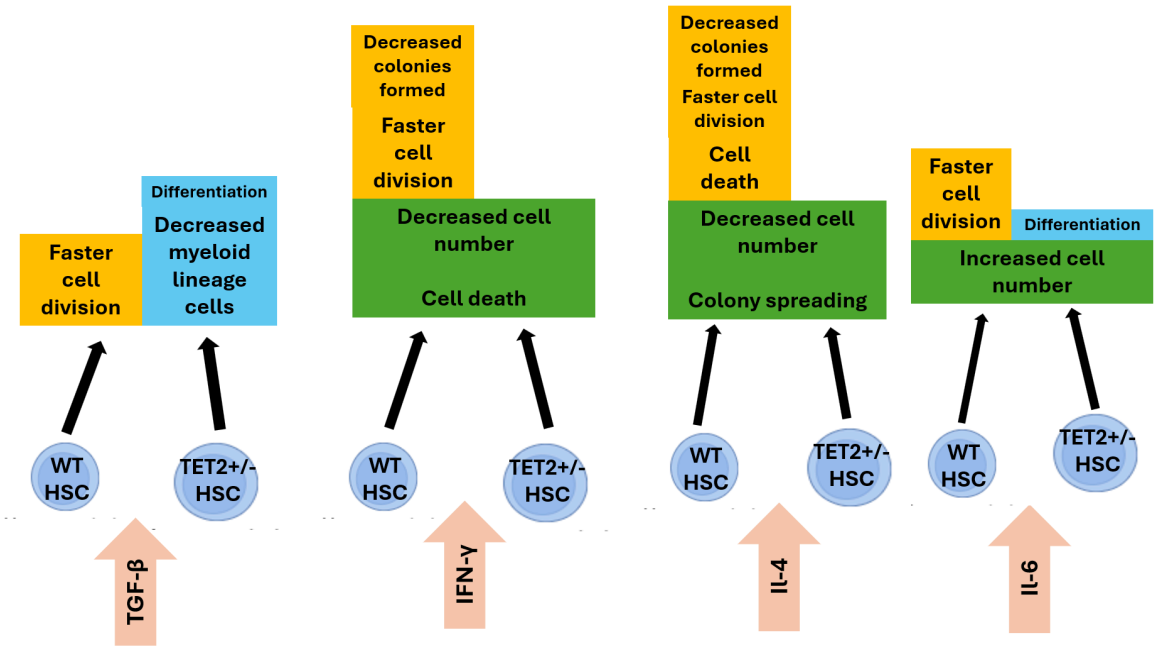

Table of Contents

List of tables

List of figures

Author's declaration

I declare that this thesis is a presentation of my own original work, and I am the sole author. This thesis has also not been previously presented for an award at this, or any other university. All sources are acknowledged appropriately as References.

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1.0 Introduction

1.1 Haematopoietic stem cells and haematopoiesis

Humans are estimated to produce more than one hundred billion new haematopoietic cells in the bone marrow each day as a result of the proliferation that occurs at each level of the hierarchy of haematopoiesis [(1)]. In the steady state across the whole skeleton, single stem cells and multipotent progenitors distribute throughout the bone marrow and are enriched around megakaryocytes. [(2)] This anatomy is maintained even after strong threats to blood cells such as systemic infection and haemorrhages [(2)].

Heterogeneity in haematopoietic stem cells (HSCs) has been described at a cellular and molecular level and also exists across the lifespan of the organism [(1)]. Understanding how these HSCs then mature into one of the full range of mature blood cells therefore also has a wide range of operational possibilities mediated by various extrinsic factors. As an example pathway, HSCs can become common myeloid progenitor cells (CMPs) through the action of TGF-β [(2)] and the CMP, following exposure to thrombopoietin (TPO), becomes a megakaryocyte which is the precursor cell for platelets [(3)]. Other pathways can also be activated. IL-5 exposure can promote the differentiation of eosinophils [(4)] whereas IL-3 can promote the development of basophils [(5)]. Erythropoietin (EPO) stimulates the differentiation of erythrocytes [(6)] whereas macrophage colony-stimulating factor (M-CSF) and IL-34 stimulate the development of monocytes which can differentiate into dendritic cells upon exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophages after exposure to IL-4 [(7–9)]. Granulocyte colony-stimulating factor (G-CSF) promotes the development of neutrophils [(10)].

In contrast to the myeloid cells described above, exposure to substances such as BMP (Bone Morphogenetic Protein) which is part of the TGF-β superfamily, can lead HSCs to differentiate into common lymphoid progenitor cells (CLPs) [(1)]. These CLPs can differentiate into T-cells after exposure to Notch [(11)], B cells following B lymphocyte stimulator (BLyS) which can become plasma cells after exposure to IL-16 [(12,13)] and NK cells through IL-15 stimulation [(14)]. Collectively this is regularly referred to as the haematopoietic hierarchy, and this is illustrated in **Figure 1**.

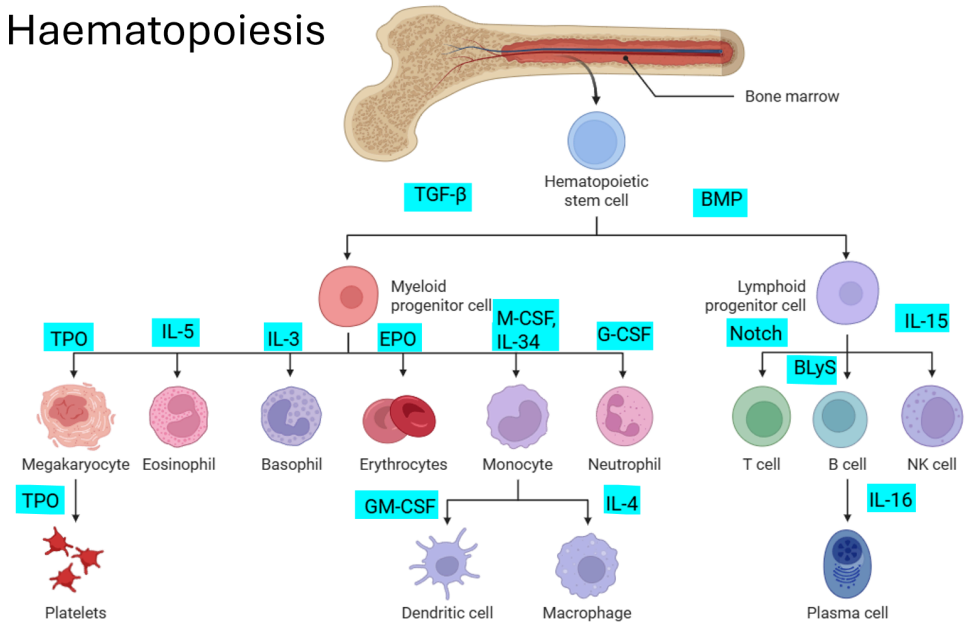


Figure 1: Haematopoiesis and the main cytokines involved

HSCs differentiate into mature blood cells through the control of multiple cytokines at various branch points. The factors listed in the diagram are just a selection and not a comprehensive list as each differentiation pathway tends to involve multiple cytokines and signalling cascades. [Created using BioRender.com]

HSCs and multipotent progenitors (MPPs) divide only occasionally and spend most of their cellular life outside of the cell cycle, in a reversible state termed quiescence or G0 [(3)]. As HSCs and MPPs largely differ in their self-renewal capacity (HSCs possess long term self-renewal capacity, MPPs do not) and lineage preferences [(3)], this places quiescence as the only fundamental common property of all HSC/MPPs during steady-state adult hematopoiesis.

Classic features of quiescence include low protein synthesis and a reliance on glycolytic metabolism and these characteristics are shared by many adult stem cells [(4)]. The maintenance of this state in HSCs requires specific metabolic and organelle functions such as a large quantity of inactive mitochondria [(5)] along with many large and inactive lysosomes [(6)].

In stem cell systems with a hierarchical organisation, it has been shown that quiescence is not a uniform state and HSCs exist in varying depths of quiescence (measured via the time a cell takes to exit this state upon receiving a mitogenic stimulus (quiescence exit: from G_0 to end of early G_1), often approximated to the time of first division ex vivo)_in order to differently and appropriately contribute to tissue

maintenance and regeneration [(7,8)]. HSCs only divide four to six times in adulthood whereas progenitors and mature cells divide much more frequently [(9,10)]. Through the use of inducible label retention assays in mice, analysis of the heterogeneity in division frequencies of HSCs under stress and homeostatic conditions has been undertaken. This allowed for the observance that the exit from quiescence of dormant HSCs takes the longest amount of time, but they possess the highest degree of long-term repopulation capacity [(11,12),(9,13–15)]. This correlation is so strong that these features can both be used as surrogate markers for the long-term repopulation capacity of an HSC. The dormant HSC is recruited into cycling only in the context of severe stress [(10)]. However, HSCs that have been activated by injury can return to dormancy and restore the proportion of dormant HSCs [(10,11)]. However it has been shown recently that by returning to quiescence, dysfunctional mitochondria permanently build up inside HSCs which helps to explain why their function can decline through division [(16)]. It has been suggested that the capacity of HSCs for self-renewal may decline with each successive division and the cells become increasingly less likely to return to quiescence [(13,14)]. Single HSCs are also able to outlast the mouse lifespan [(13)].

A causal relationship between the cell cycle and subsequent cell fate decisions has been a widely researched topic in stem cell biology. The position in the cell cycle has been shown to potentially influence how a cell responds to external stimuli [(17)]. It has also been suggested that the duration of a particular cell cycle phase may also influence cell fate [(18)]. In ES cells, the differentiation route that cells take is causally related to the duration of G_1 [(19–21)]. This effect is also observed in neural stem cells when G_1 is lengthened [(22,23)]. The presence and purpose of these mechanisms in HSCs are still not conclusively demonstrated. Some effects of CDK4 and cyclin D1 overexpression have been observed in human HSCs upon xenotransplantation but this has not been further supported by experiments in vitro [(24)]. It is difficult to confirm whether the slight bias in lineage differentiation observed in these cells is in response to cell cycle length alterations as there is a high chance that other factors may play a role due to the pleiotropic effects of CDKs and cyclins [(4)]. Heterogeneity in the quiescence states of HSCs has been shown to be dependent on the varying levels of CDK6 expression [(12)]. This is because CDK6 is able to function as a master regulator of the duration of exit from quiescence and its absence from LT-HSCs limits their divisions and as a result can preserve the integrity of the HSC pool for the long-term [(12)]. It is possible that this delay in exit from quiescence of LT-HSCs can be important to the cell fate decisions of these cells on exposure to stress [(12)]. In addition, the presence of CDK6 in ST-HSC is sufficient to facilitate their division in addition to their activation in response to mitogenic stimuli [(12,25)]. In order to further the above research, transgenic mice that lack either CDK4 or CDK6 in adult hematopoiesis have recently been generated [(26)]. These mouse models will allow for the functions of CDK4 and CDK6 in a tissue-specific manner to be elucidated [(26)]. Distinguishing between functionally defined HSC and MPP populations through global gene expression

analysis allowed for the identification and subfractionation of the most primitive subpopulation of long-term repopulating cells within the phenotype human LT-HSC (CD49f+ CD90+) compartment [(1)]. This population (purified as $CD34^{hi}$iCD38–CD45RA–CD90+CD49f+$CLEC9A^{lo}$) was also characterised by short quiescence exit and higher CDK6 levels than LT-HSCs or $CLEC9A^{hi}$ LT-HSCs ($CD34^{lo}CD38^{-}CD45RA^{-}CD90^{+}CD49f^{+}CLEC9A^{hi}$) [(2)].

There is considerable cellular heterogeneity within the HSC compartment [(27)]. Subsets of HSCs differ in their lineage outputs following transplantation and their responses to external signals [(27,28)]. After transplantation, HSCs were able to be separated into 16 different groups based on their repopulation kinetics determined by measuring the composition of blood on a bimonthly basis [(28)]. Secondary transplants demonstrated that these subsets of HSCs were conserved as the secondary hosts exhibited the same repopulation kinetics as the original HSC [(29)]. Further research allowed for the identification of ‘myeloid-biased’, ‘lymphoid-biased’ and ‘myeloid-lymphoid balanced' HSCs based on the predominant lineages produced through transplantation experiments [(30,31)]. The distribution of these lineage-biased subsets of HSCs has also been shown to change with age as mice older than 38 weeks show an increased proportion of myeloid-biased HSCs [(30,32)]. In addition, these subsets can express different cell-surface markers and exist in different states of the cell cycle [(27,33)]. The intrinsic cell cycle activity of each HSC is also thought to contribute to heterogeneity as dormant HSCs can cycle only once every 149-193 days and active HSCs can cycle once every 28-36 days [(33,34)]. This status can also alter how responsive the HSCs are to injury [(33)].

Recent studies using single-cell gene expression profiling have provided complementary molecular evidence of distinct haematopoietic stem cell (HSC) states, and this has highlighted that wild-type HSCs are a heterogeneous mix of cells that are in distinct proliferative and self-renewing states [(35)]. Gene expression analysis has subsequently progressed to the global characterisation of the transcriptome in single HSCs which develops our understanding of haematopoiesis and its hierarchical organisation [(36)]. Linkages between gene expression and cell-state changes can be inferred from reference datasets in haematopoiesis [(37)].

It has been shown that malignancy can result from disturbances in the haematopoietic balance, and this leads to the production and expansion of HSC clones that possess differentiation and/or proliferation abnormalities [(38–40)]. The challenges associated with the longevity of HSCs include the accumulation of genotoxic, proteotoxic, and oxidative stress. These potentially cannot be distributed to progeny as quiescent HSCSs do not undergo cell division [(4)]. In order to overcome these challenges, damaged HSCs are likely culled to mitigate the risk of leukemic transformation, and this would help to maintain the highest possible standards of organelle, proteostatic and genomic integrity [(4)].

1.2 The regulators of haematopoiesis

It has been shown that LT-HSC numbers and function can be directly and indirectly regulated by local niche cells, as shown in **figure 2** [(41)]. The spatial organisation of cells in a tissue has the power to dictate the behaviour of the cells and profoundly impact their function [(42)]. To maximise the quantities of functional HSCs in culture, small molecule inhibitors aimed at removing differentiation factors, limiting the accumulation of damaging agents and/or retaining quality control mechanisms associated with quiescence are important [(43–47)]. These studies all demonstrate that it is crucial to preserve the entire underlying molecular network, stress response regulation, and macromolecular organelle biology associated with quiescence that is present in vivo when culturing HSCs.

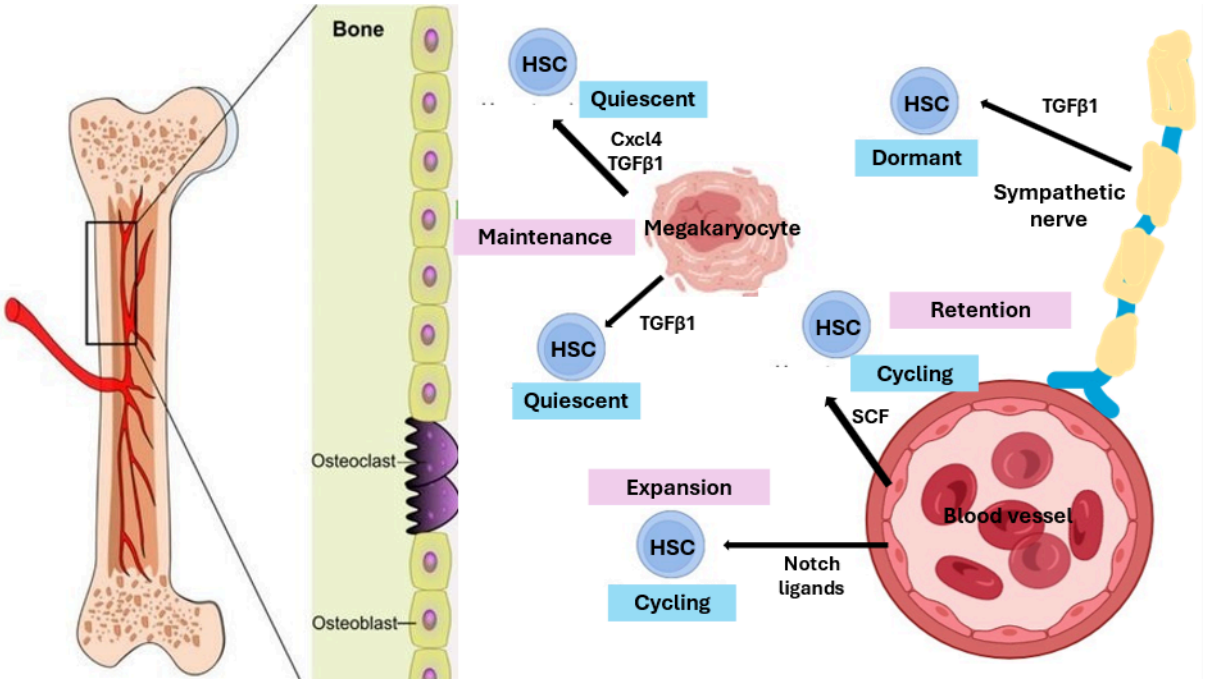


Figure 2: The haematopoietic stem cell niche

HSCs can exist in a range of cellular states which can be governed by the cells and molecules of the stem cell niche. There are two main types of niche, the endosteal niche which is near the bone surface and primarily promotes HSC quiescence, and the perivascular niche which is near the blood vessels and primarily promotes HSC mobilisation and differentiation. Both types of niche contain osteoblasts, endothelial cells, and other stromal cells but the specific types and ratios of these cells varies throughout the bone. HSC subsets may even reside in different niches and the niche is not static and can change depending on the specific needs of haematopoiesis. [Modified from (48)]

Several cell types have been implicated in regulating HSCs such as macrophages, megakaryocytes, Leptin receptor positive mesenchymal stromal cells and endothelial cells along with sympathetic and nociceptive nerve fibres [(49–56)]. And many of the molecules they produce have been the subject of numerous studies. Some key factors (such as SCF, Cxcl12, and Pleiotrophin) that HSCs require to be maintained in the bone marrow are synthesised by Leptin receptor positive mesenchymal stromal cells and endothelial cells [(49–52)]. Macrophages regulate HSC quiescence by secreting TGF-β [(53)]. Megakaryocytes have also been shown to affect HSC quiescence by secreting Cxcl4 and TGFβ1[(54)]. TGF-β ligands signal through serine/threonine kinase receptors and following ligand binding and receptor phosphorylation, the SMAD transcription factor family signalling circuit becomes activated [(57)]. TGF-β is known as one of the most potent inhibitors of HSC growth and proliferation in vitro [(58–61)]. It maintains HSC dormancy and quiescence and the neutralisation of TGF-β in vitro has been shown to promote the release of early HSPCs from quiescence [(62–64)]. An interesting finding is that TGF-β appears to induce different responses based on the specific subtype of HSCs exposed to it [(65)]. Specifically, the proliferation of myeloid biased HSCs is promoted whereas the proliferation of lymphoid biased HSCs is inhibited [(65)].

It has also been shown that ablation of sympathetic and nociceptive nerve fibres can increase the number of HSCs in the bone marrow [(55,56)] but neither are known to influence HSCs and haematopoiesis under normal conditions. The release of Calcitonin Gene-Related Peptide, a protein which activates receptors that are expressed by HSCs, by nociceptive nerve fibres is known to promote HSC mobilisation [(66)]. Regulation of the post-translational processing and activation of latent TGF-β by non-myelinating Schwann cells is also known to promote HSC quiescence [(55)].

The bone marrow is also capable of adjusting blood cell production in response to different physiological insults [(67)]. For example, after a haemorrhage, HSCs proliferate in an erythrocyte biased manner to recover the erythrocyte populations that were lost. [(68)]. These reactive responses have been well characterised but primary or direct changes to the HSPC compartment after infection have not yet been considered properly.

1.3 Haematopoiesis in response to stress

The bone marrow is capable of adjusting blood cell production in response to different physiological triggers. In the steady state across the whole skeleton, single HSCs and multipotent progenitors distribute throughout the bone marrow and are enriched around megakaryocytes [(54)]. On the other hand, lineage-committed progenitors have been shown to be recruited to blood vessels [(67,69)]. In the blood vessels, they form part of lineage-specific microanatomical structures which are

composed of progenitors and immature cells [(67,70,71)]. These structures function as the production sites for each of the major blood cell lineages [(67)]. This anatomy is maintained even after strong threats to blood cells that require responses such as haemorrhages, systemic bacterial infections and exposure to granulocyte colony-stimulating factor (G-CSF) treatment [(67)].

Despite progress in haematopoiesis research, the anatomy/organisation of haematopoiesis in normal HSCs compared with HSCs experiencing stress remains unclear. This lack of research is largely because current techniques do not allow for simultaneous imaging of most types of progenitors and their daughter cells [(67)]. This limit also prevents *in situ* analysis of haematopoiesis. Solving this methodological problem will be essential for elucidating the differences in cell behaviour during differentiation, defining relationships between parent and daughter cells and identifying the cells and structures that enable the differential normal and stress haematopoiesis [(67)]. Recently reporter mice have been developed to contribute to solving some of these issues [(11,72,73)]. For example, Gprc5c-controlled reporter mice were developed to allow for the tracking of dormant or highly quiescent HSCs, clusterin (Clu)-green fluorescent protein (GFP) reporter mice have allowed for the identification of HSC subsets associated with aging and the Fgd5 reporter mouse can facilitate the identification of HSCs in expansion cultures [(11,72,73)]. Omics techniques have also shown promise for understanding haematopoiesis and stress responses in detail [(74)]. The use of CloneTracer which adds clonal resolution to single-cell RNA-seq datasets has allowed for the identification of cell-surface markers that are specifically dysregulated in acute myeloid leukaemia and revealed the differentiation landscape of HSCs [(74)]. Long-read single-cell transcriptomics and proteogenomics has allowed for understanding the consequences of somatic mutations in a cell-type-specific manner which can further develop our understanding of haematopoiesis in response to different stresses [(75)].

1.3.1 Haematopoiesis and aging

The deterioration of HSCs and the bone marrow microenvironment characterises the functional decline of haematopoiesis with age [(76)]. This has been shown to lead to reduced regenerative ability, impaired immune cell production and function, an increased risk of anaemia, autoimmune diseases, increased susceptibility to infection, and haematological malignancies [(77)]. Aged HSCs demonstrate reduced colony forming efficiency, delayed proliferation and reduced multipotency with myeloid lineage bias compared to young HSCs [(78,79)]. Cytopenias such as anaemia, neutropenia and thrombocytopenia are common in older people [(80–82)]. The risk and incidence of myelodysplastic syndrome and bone marrow failure also increase with age [(83,84)].

HSCs have been shown to not decline in number and actually expand with age in mice with one study linking this to a greater preservation of sinusoids during aging [(85,86)]. This increased number of HSCs in older mice is countered with decreased function [(78)]. The loss of p16 (Cdkn2a), an important tumour suppressor, results in HSC pool expansion only in old mice which could potentially link p16 with the defective haematopoiesis shown in older mice [(87–90)]. Aging has also been shown to specifically impair lymphoid-lineage differentiation of HSCs without impacting myeloid-lineage differentiation [(78)].

It is now generally accepted that quiescence is an important property of HSCs and as such, mutations that promote proliferation would likely decrease HSC function [(91)]. Mutations that sustain excessive proliferation do adversely impact HSC function [(91)]. For example, Gfi1 under normal conditions limits proliferation to preserve the functional integrity of HSCs [(92)]. Knockout of genes such as Irgm1 or Adar1, which negatively regulate IFN-γ signalling in HSCs to protect them from autophagy and promote proliferation, have only very minor impacts on baseline homeostasis of the blood but can have a severe impact after stress [(93–95)]. Further knockout mouse models have identified Evi1 and cMpl as important for long-term HSC function, with overexpression of or mutations in both of these genes implicated in haematological malignancies [(96–98)]. The CDK inhibitor p57 has been shown to have minimal impact on the developmental stages of haematopoiesis but in later life, when HSCs became more quiescent, detrimental effects were observed [(99,100)]. The mitotic activity of HSCs has been shown to be dependent on the length of telomeres, as it is in other somatic cells, and this decreases with age [(101)]. A correlation between the self-renewal capacity of HSCs and telomerase activity has been suggested [(102)]. It has been suggested that the most primitive HSCs may only divide 4-5 times and the phenotype associated with age may only be present after the fifth division [(13)]. This implies that with each division of each HSC, their function may decline [(103)].

Studies into the genes responsible for HSC function identified potential targets of epigenetic dysregulation that can lead to the decline in function with age [(91,104)]. Research into these epigenetic changes associated with age identified genes involved in inflammatory and stress responses as the majority of genes that are upregulated and those involved in chromatin regulation and DNA repair as the majority of genes that are downregulated in older mice [(104,105)]. The upregulation of genes involved in inflammation and stress responses suggests that a proinflammatory microenvironment is likely a feature of the aging bone marrow [(104)]. Some of the changes in haematopoiesis that occur with age are therefore likely to result from the increased levels of inflammatory growth factors such as Interferons, IL-1β, IL-6, TNFα, and Rantes [(106–109)]. The genes involved in chromatin regulation and DNA repair (such as Xab2, Rad52, Polb, Lmna, Smarca4 and Smarcb1) being upregulated in older mice support the idea that epigenetic dysregulation impacts aging HSCs as a wide range of genes could be affected as a

result [(104)]. Upregulation of these genes seems to occur mainly around mid-way through life and as a result could underlie the inappropriate expression of a wide range of additional genes that is associated with aging [(104)]. In addition, these epigenetic changes could drive the defective function of HSCs which characterises aging [(104)].

1.3.2 Haematopoiesis and infection

Pathogens have also been shown to influence haematopoiesis. Studies have shown that E. coli infections stimulate expansion and mobilisation of LSK cells [(110)] LSK cells are also stimulated in models of systemic polymicrobial, viral, and candida infection [(111,112)]. On the other hand, alternative pathogens such as Ehrlichia chaffeensis or Anaplasma phagocytophilum have been shown to diminish HSPC numbers through anaemia, thrombocytopenia, and a reduction in cellularity of the bone marrow in infected mice [(113,114)] In these studies, the number of committed progenitors were reduced in the bone marrow and infectious particles were not detected suggesting that the HSPCs themselves were not infected and were instead responding to systemic infection. Listeria monocytogenes infection was shown to result in more primitive progenitor populations and selective expansion of the monocyte lineage [(115)]. Murine cytomegalovirus infection has been shown to suppress the cellular proliferation of myeloid progenitors [(116)]. Collectively, these studies demonstrate that, depending on the type of infection, the immune system might become biased to a myeloid or lymphoid response based on the responses of the HSPCs. In these models, however, effects were studied in committed progenitors not on more primitive cells such as HSCs.

In a polymicrobial sepsis model that unfortunately didn't compare engraftment efficiencies of HSCs between infected and uninfected mice, the LSK progenitor compartment expanded rapidly but there was reduced stem cell activity and differentiation capability [(117,118)]. HSCs are known not to become infected themselves, in this context, so the numerical and functional changes seen in the cells during infection are potentially mediated by inflammatory signals generated by the progenitor cells and more mature immune cells [(119,120)]. In addition, LPS from E. coli acts on LSK cells through TNFα and NFκB signalling [(110,121–123)]. In a similar manner, vaccinia virus and candida albicans infections both lead to LSK expansion via a MyD88-dependent pathway which shows a role for TLR signalling in this process [(111,112,124)]. However, LT-HSCs have been shown to become infected with leishmania parasites at relapse [(125)]. These cells, as hosts for leishmania, have also been shown to play an important role in treatment and oxidative stress resistance through upregulation of TNF/NF-κB and RGS1/TGF-β/SMAD/SKIL signaling pathways [(125)].

IFNs have emerged as an important component of HSC regulation [(126,127)]. IFNs were originally discovered as factors that impair viral replication [(128)] The IFNs are subdivided into two groups: type I IFN which includes IFNα and IFNβ which both bind to the IFNα receptor, and the type II IFNγ which binds to the IFNγ receptor [(129,130)]. The type I IFNs are induced by viral infection and can be synthesised by most types of cells that have been infected by viruses [(131)]. However, type II IFN is induced by mitogenic or antigenic stimuli to act against a wide variety of intracellular pathogens but can only be produced by some immune effector cells such as NK and T cells [(132)]. In addition, IL-4 is type one cytokine that can be released from inflammation, fibrosis and allergic reactions and it can act on a range of cell types but has been shown to induce apoptosis in HSCs [(133)]. IL-4 has also been shown to influence differentiation by downregulating HSPC differentiation genes and inhibiting megakaryocyte differentiation [(133)].

1.4 Isolation of haematopoietic stem and progenitor cells

Pioneering work from the Weissman and Nakauchi labs showed that LSK (lineage negative, Sca-1 and cKit positive) cells defined immunophenotypically contain most HSC activity in the bone marrow of the mouse. [(134–136)]. One issue with many early HSC studies is the application of the LSK designation to define the HSC compartment even though this compartment includes many different cell types such as committed progenitors [(137)]. The phenotypic identification of HSCs in the context of stress such as inflammation or infection is also difficult due to marker lability, thus requiring new functional validation of flow cytometric findings and making studies more challenging. An example of this is Sca-1 which is known to be induced by inflammatory signals such as interferon gamma, interleukin-15 and interleukin-12 even on the surface of lineage-committed cells such as NK cells or in a range of different tissues [(138,139)]. Markers such as CD117 (also known as c-kit) is commonly found on the surface of many different types of stem cells and is activated by stem cell factor (SCF) [(140–142)]. A further marker with variable expression patterns is macrophage-1 antigen (MAC-1) as this has been found to have briefly higher expression levels during the early reconstitution phase of HSC transplantation and as a result could serve as a marker to identify functionally superior quiescent HSCs in mice [(143)]. In humans, CD38 is known to have variable expression levels on HSCs as certain serum factors have been shown to stimulate its expression [(144)].

Common lineage markers for HSC cultures include CD11b and Ly6G. CD11b is used in the lineage negative gating of LSKs and ELSKs (EPCR positive LSKs) because it is common on the surface of many myeloid-lineage HSPCs and committed mature cells such as macrophages, neutrophils and dendritic cells [(145–148)]. The same is true for Ly6G as it is also present on many myeloid-lineage HSPCs and committed

mature cells such as neutrophils and eosinophils [(149–155)]. Further lineage marker combinations used for more definitive isolation of HSPCs from whole bone marrow include CD5, CD11b, CD19, B220 (also known as CD45R), Ly6G/C(Gr-1), TER119 [(156)]. CD5 is expressed on lymphocyte precursors and all mature T-cells in addition to a subset of mature B-cells while CD19 and B220 are expressed on almost all B-lineage cells [(157–159)]. Gr-1 is the combination of two commonly expressed myeloid lineage markers, Ly6G and Ly6C and as such it can mark neutrophils, monocytes, certain subsets of macrophages and dendritic cells, as well as myeloid-derived suppressor cells (MDSCs) [(153,160)]. TER119 is included in panels and isolation kits as well due to the fact that it is able to identify and exclude many of the late stages of murine erythroid lineage cells [(161)].

Further markers used to identify haematopoietic cells within the bone marrow include CD49b, ROBO1 and PrP(C) [(162–164)]. CD49b has been shown to mark multipotent HSCs with a lymphoid bias and reduced self-renewal ability [(162)]. When the expression of ROBO1 is reduced, Myelodysplastic syndrome (MDS) symptoms can result as early erythropoiesis is blocked [(163)]. Cellular prion protein (PrP(C)) is expressed on HSCs, granulocytes, T and B lymphocyte NK cells, platelets, monocytes, dendritic cells, and follicular dendritic cells [(164)].

The next leap forward for immunophenotyping was the discovery of signalling lymphocyte activation molecule (SLAM) to discriminate between HSCs and their downstream progeny [(165)] In addition, the endothelial protein C receptor was discovered to only be expressed on the surface of HSCs [(166–168)]. An important consideration when using markers for flow cytometry is that they do show fluctuations in their expression as a result of external stimuli such as cell cycle status and motility [(127,169)]. However, EPCR has been shown to not be as sensitive to those differences and as such is a more reliable marker for HSC identification [(167)]. This makes ELSKs and ESLAM cells better denominations of stem cells in flow cytometric analyses compared to alternatives such as LSK.

Many studies have also confirmed that ESAM is uniformly expressed in all HSCs. [(170–172)]. ESAM is also uniformly expressed in subsets of multi- or oligopotent HSPCs [(67)]. It is consistently absent in all lineage-committed progenitors [(67)]. This can make it a useful marker when combined with others for isolating HSCs. Procr, which encodes EPCR, and Esam are both known to be expressed by immunophenotypically-defined LT-HSCs from a mouse embryo at embryonic day 14.5, specifically from the fetal liver [(173)]. This can allow for alternative gating strategies to identify HSCs.

1.5 Haematopoietic stem cell divisional kinetics

HSCs and MPPs divide only occasionally and spend most of their cellular life outside of the cell cycle, in a reversible state termed quiescence or G_0 [(4)]. Quiescent HSCs maintain their stress response pathways to ensure protein quality remains high [(174,175)]. Across a range of tissue stem cells, it has been found that the most immature stem cells reside in a deeper quiescent state than their progeny [(176)]. The levels of CDK4 and CDK6 proteins are a good reporter for the depth of quiescence [(176)]. The activation of these proteins is the key trigger for cells to enter G_1 regardless of whether they have just divided or been quiescent for a long time [(176)]. The activity of these proteins sustains the hyperphosphorylation of the retinoblastoma protein in early G_1 and this is essential for a cell to pass the restriction point in order to commit to division [(176)]. Quiescent long-term HSCs contain undetectable levels of CDK6 protein whereas quiescent short-term HSCs contain higher levels of CDK6 protein to facilitate more efficient cell cycle entry following mitogenic stimulation [(11,12)]. The levels of CDK4/6 activity within a single cell have been shown to be critical for determining the probability of reaching the restriction point [(177)]. This therefore allows the protein levels to potentially help serve as a marker for differential comparison of ST-HSCs and LT-HSCs. It is also possible that LT-HSCs may require a stronger/longer mitogenic stimulus to divide.

To control HSC pool size and regulate tissue regeneration, it is important to maintain distinct depths of quiescence in LT-HSCs and ST-HSCs along with the associated division kinetics [(12)]. Quiescence and the associated limit of divisions of LT-HSCs allows for the maintenance and regulation of healthy haematopoiesis even towards later life [(12)]. Whereas, the ability of ST-HSCs to enter the cell cycle to produce the mature blood cells as required allows the body to rapidly respond to injuries and infections [(12)].

In response to injuries, HSCs in mice are able to divide faster if necessary after successive challenges through their access to an mTORC1-high "G_{alert}" state [(7)]. This state is not the same as the ST-HSCs' primed quiescent state which demonstrates minimal levels of mTORC1 activity [(12)]. The molecular mechanisms involved in the drive toward and maintenance of this state in HSCs have not yet been studied but my own project may aid in identifying which cytokines potentially have an impact on stem cell fate/state adding to recent evidence that mouse HSCs possess long-term epigenetic memory of inflammatory signals by helping to identify which signals influence stem cell state (division or "dormant") [(178,179)]. Through the use of inducible label retention assays in mice, analysis of the heterogeneity in division frequencies of HSCs under stress and homeostatic conditions has been undertaken. This allowed for the observation that quiescence exit for dormant HSCs takes the longest amount of time, but dormant HSCs possess the highest degree of long-term repopulation capacity [(9,11–15)]. HSCs that have been activated by injury can return to dormancy and restore the proportion of dormant HSCs [(10,11)]. However it has been shown recently that by returning to quiescence, dysfunctional mitochondria permanently build up inside HSCs which helps to explain

why their function can decline through division [(16)]. The alterations in the balance of quiescent states associated with aging or disease still are not yet properly characterised. The niche-driven effects, life histories of infection/inflammation and age-related clonal haematopoiesis are all important components to consider for understanding the responses of HSCs to age [(180,181)].

A challenge that remains for HSC expansion is that prolonged culture ex vivo leads to a net decline in HSC long-term repopulation capacity as a result of division becoming biased towards differentiation [(182–185)]. There are thought to be many causes of this decline, but it is most likely highly dependent on the culture medium composition and culture duration [(186,187)]. The work of Wilkinson et al., optimised the components of the media and incubator conditions for 236–899-fold murine HSC expansion [(188)]. This highlighted that after sorting, culture plates should be immediately placed in a sterile and humidified tissue culture 37°C incubator with 5% CO_2 and 20% O_2 and should only be removed for no longer than 5-15 minutes if absolutely necessary [(189)]. The protocols [described in (189)] have been able to support the long-term expansion of functional murine HSCs [(45)].

1.6 Haematological malignancies and driver mutations

The TET proteins are α-ketoglutarate and Fe^{2+}-dependent enzymes which change the epigenetic status of DNA by oxidising 5-methylcytosine to 5-hydroxymethylcytosine [(190–194)]. The TET proteins, as shown in **figure 3**, are expressed in many cell types including HSCs and so their impacts on HSC differentiation have been widely studied [(190,195)]. In many patients with a range of myeloid malignancies, loss of function TET2 mutations have frequently been observed [(196)]. TET2 has subsequently been found to be a critical regulator of self-renewal and differentiation of HSCs in mouse models [(196)]. While most TET2 mutations associated with myelodysplastic syndrome (MDS) and chronic myelomonocytic leukaemia (CMML) lead to the loss of the entire protein, some are missense mutations which involve either the catalytic or non-catalytic domains [(197,198)]. Deletion of TET2 in mice has been shown to increase HSC self-renewal and bias differentiation towards the monocytic/granulocytic lineages which leads to the CMML-like phenotype [(196,199,200)]. Also, complete loss of TET2 has been linked to lymphoid disorders such as angioimmunoblastic T lymphomas, mantle cell lymphomas and diffuse large B cell lymphomas [(201–207)].

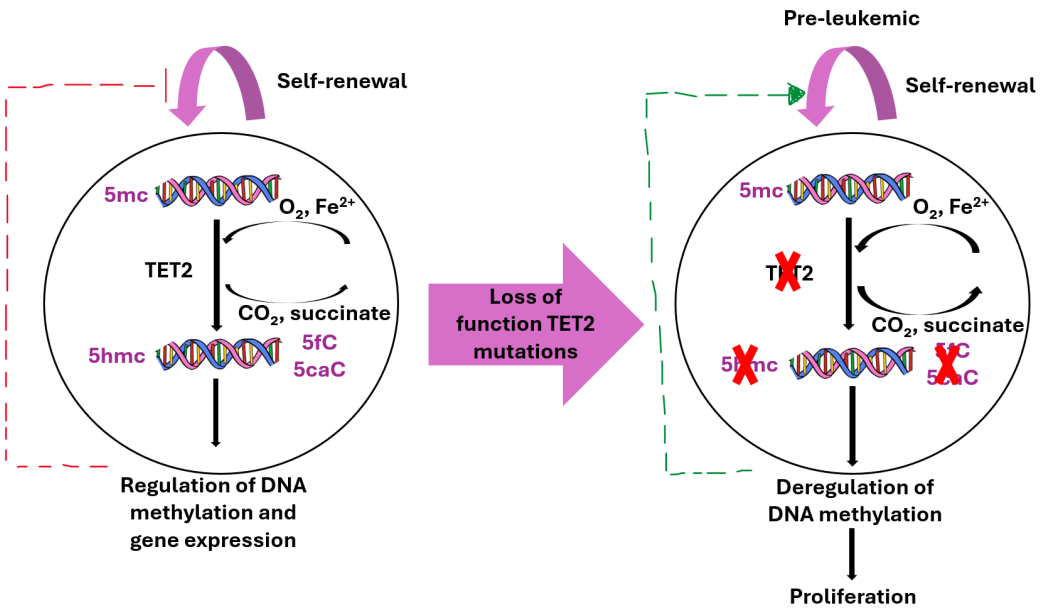


Figure 3: the consequences of TET2 loss of function mutations

TET2 mutations are frequently observed in a range of myeloid malignancies because the TET2 gene encodes a demethylase enzyme involved in regulating genes involved in proliferation and self-renewal. As a result of loss of function TET2 mutations, 5-methylcytosine (5mc) cannot be oxidised to 5-hydroxymethylcytosine (5hmc) which can lead to myeloid malignancies and myeloproliferative neoplasms. [Modified from (196)]

It is the C-terminus of TET2 that is responsible for its catalytic activity and can result in DNA hydroxylation [(190)]. TET2 is also involved in the formation of chromatin regulatory complexes with histone deacetylases (HDACs) and O-linked N-acetylglucosamine transferase (OGT) which regulate transcription and histone modifications [(208–211)]. Many previous attempts to elucidate the roles of Tet2 in haematopoiesis use knockout mouse models, either conventional or conditional, which lack the entire Tet2 protein and so fail to distinguish between its catalytic and non-catalytic requirements in a physiologically relevant context. New non-catalytic requirements for Tet2 in the HSPC homeostasis and in incorrect lymphopoiesis were demonstrated [(196)]. Base substitutions, splice site mutations and out-of-frame insertions/deletions within TET2 are observed in a range of myeloid malignancies such as MDS, MPN, CMML, AML and sAML. [(197,212–218)]. These studies demonstrated the missense mutations that were associated with leukaemia hindered the enzymatic activity of TET2 [(193)].

The associated TET2 deficiency has been shown to lead to reduced 5-hydroxymethylcytosine levels across the genome and alter the size of the

haematopoietic stem/progenitor cell pool in a cell-autonomous manner as this research also demonstrated that TET2 likely has a role in homeostatic regulation, but this role differs between HSCs and ES cells which implies that the cellular context can have a role in regulating/controlling TET2 function [(196)].

In vitro TET2 deficiency has been shown to delay HSC differentiation and prioritise monocyte/macrophage lineage development [(196,219)]. The data gathered by others show that Tet2 has an essential role in regulating the expansion and function of HSCs, and additional data suggests that this may be due to Tet2 controlling 5hmC levels at genes that are relevant for self-renewal, differentiation and proliferation [(196)]. Studies involving shRNA- mediated knockdown of TET2 in mouse HSPCs showed a potential role for Tet2 in controlling myeloid differentiation and the homeostasis of HSPCs [(193,220)].

1.6.1 Haematological malignancies and cytokines

Characteristics of the responses of HSCs to interferon gamma are altered in TET2 knockout HSCs [(221)]. Single-cell RNA sequencing has demonstrated that murine TET2 knockout myeloid progenitors are enriched for IFN-induced gene expression even at baseline [(222)]. As IFN-γ has an important role in the activation of myeloid cells, the response of TET2 knockout cells is heightened and nearly all cell types are able to have the phosphorylation of STAT1 induced by IFN-γ [(221,223)]. The responses to IFN-γ stimulation were most pronounced in cells of the monocyte and granulocyte lineage though [(221)]. These responses are also known to be heterogeneous within the HSPC compartment [(221)]. It will be important to further investigate the responses of TET2 mutant HSCs specifically to IFN-γ compared with wildtype HSCs.

IL-4 levels are also known to be increased in response to TET2 mutations, and this could explain why thrombocytopenia is common in AML [(133)]. As a result of the inflammatory environment induced by TET2 mutations, the responses of TET2 mutant cells to cytokines have been studied in various contexts. Tet2 deficiency was found to not alter the impacts of IL-4 on macrophages significantly [(224)]. However, responses to many other cytokines such as IL-6 have been shown to be altered in macrophages by Tet2 deficiency [(224)]. It is therefore worth investigating whether the responses to IL-4 of TET2 mutant HSCs are any different to those of wildtype HSCs.

The role of TGF-β in the context of various malignancies has been widely studied. It has been shown to have a substantial impact on the initiation, progression and metastasis of malignancies in nearly every type of cell [(225)]. TGF-β has also been shown to suppress the tumour inhibitory effects of T-cells, NK cells, neutrophils, monocytes and macrophages [(226–228)]. The expression of TGF-β has also been found to be upregulated in many human malignancies [(229,230)]. TGF-β is also involved in haematological malignancies too as in leukaemias, myeloproliferative

disorders, lymphomas, and multiple myeloma resistance to the homeostatic effects of this cytokine can be developed [(231)]. As TET2 mutations are also key components of the pathogenesis of malignancies such as AML, it is worth further investigating the responses of TET2 mutant HSCs to TGF-β in order to determine which effects are altered or even non-existent in these cells. As TET2 mutant HSCs are known to be biased towards the myeloid lineage, they could undergo increased proliferation and self-renewal in response to TGF-β, as this cytokine is known to promote proliferation of myeloid lineage cells as described previously [(232)].

A further cytokine with relevance in the context of haematopoiesis and TET2 mutations is IL-6 as it is known to be in the plasma at greater quantities of TET2 mutant mice compared with wildtype [(233)]. IL-6 is able to induce hyperactivation of Shp2-Stat3 signalling, which results in increased expression of a novel anti-apoptotic long non-coding RNA (lncRNAs), Morrbid, in Tet2 knockout myeloid cells and HSPCs [(234)]. In Tet2 mutant mice, pharmacologic inhibition of Shp2 or Stat3 or genetic loss of the lncRNA Morrbid rescues the inflammatory-stress-induced abnormalities often seen in their HSPCs and mature myeloid cells, including clonal haematopoiesis [(234)]. This demonstrates the importance of IL-6 to the inflammatory state of TET2 mutant mice and it would be interesting to know whether the responses to this cytokine are comparable to wildtype for TET2 mutant HSCs.

As TET2 mutant HSCs are more capable of retaining their “stem” characteristics, their divisional kinetics in response to inflammatory cytokines could be quite different to wildtype. Tracing the division patterns of TET2 knockout HSPCs ex-vivo in real time demonstrated that these cells underwent more symmetric self-renewal [(235)]. This characteristic may impact the divisional kinetics of $TET2^{+/-}$ HSCs in addition to their cell fate decisions as HSCs divide slower than progenitors. It will be useful to determine whether cytokines are able to cause $TET2^{+/-}$ HSCs to differentiate and divide faster as a result like wildtype HSCs.

Furthermore, Vitamin C, which boosts TET enzymatic activity, has been shown to have therapeutic benefit in MDS and CMML disease in Tet2 haploinsufficient mice [(236,237)]. This shows the potential of targeting the TET proteins in haematopoietic malignancies. In order to facilitate this, it is important to understand the complete functions of these proteins and how mutations impact each of these functions.

The threat of lineage-biased differentiation of HSCs is made apparent in different types of leukaemia when patients primarily produce a single cell type as opposed to the full range of blood cells [(238–243)]. The persistence of the leukemic stem cells despite treatment permits recurrence of disease and this is why there is a very poor 5-year survival rate overall for acute leukaemia. The TET2 heterozygous HSCs provide a good model for many myeloid malignancies, as described previously, and so it is important to determine whether these HSCs respond to inflammatory signals in the same way as healthy wildtype HSCs. In addition, if any differences in

responses are detected, whether these could be exploited in order to improve patient prognosis is important work to follow.

1.7 Aims and hypotheses

In this thesis we set out to study the cytokine response of wildtype and TET2 mutated HSCs to gain insight into whether specific pathways might contribute to explaining the differing cell fates between mutant and non-mutant HSCs.

As a result of the known myeloid malignancies that associate with TET2 mutations, it would be reasonable to expect that cultures initiated with $TET2^{+/-}$ cells would retain a greater proportion of stem cells despite the treatments and would produce more myeloid cells regardless of the type of inflammatory cytokine. We also hypothesise that Tet2 deficient HSCs will be resistant to the death and differentiation promoted by effector cytokines such as IFN-γ and IL-4, and also resistant to the regulation induced by regulatory cytokines such as TGF-β. As a result, in both instances Tet2 deficient HSCs would possess a competitive advantage over wildtype HSCs.

2.0 Materials and methods

2.1 Mice

All mice (wildtype and TET2+/-) were bred in house at the Biological Services Facility at the University of York, UK [(200)]. Animals were housed in individually ventilated cages (IVC) and provided with sterile food and water ad libitum. All mice were kept in specified pathogen-free conditions, and all procedures performed according to the United Kingdom Home Office regulations, in accordance with the Animal Scientific Procedure Act 1986.

BM was obtained from wildtype and $TET2^{+/-}$ mice aged from 8-12 weeks old (detailed in supplementary) through removing the iliac and femur and then crushing them using a pestle and mortar. Ammonium chloride (STEMCELL technologies, #07850) at a concentration of 20 µg/mL was then added to the bone marrow in order to lyse erythrocytes.

2.2 Isolation of HSCs

The BM samples underwent lineage depletion using EasySep Mouse Hematopoietic progenitor cell isolation kit (STEMCELL Technologies, #19856A) followed by EasySep™ Streptavidin RapidSpheres™ (STEMCELL Technologies, 50001). Then, cells were sorted using the ESLAM panel (EPCR, CD48 and CD150) with the addition of Sca-1 and CD45. HSCs were sorted as EPCR, CD150, CD45 and Sca-1

positive and CD48 negative [(133,211,244–246)]. The live/dead stain used was 7-Aminoactinomycin D (7AAD) at a concentration of 1 ug/mL. (Invitrogen, #A1310). The gating strategy is shown in **Figure 4**. The cells were then individually sorted into single wells of a 96-well culture plate (Greiner Bio-One, #675 180) using the MoFlo Astrios cell sorter (Beckman Coulter life sciences) which is equipped with four lasers, CytoFLEX SRT Benchtop Cell Sorter which has four lasers or BD FACSDiscover™ S8 Cell Sorter which has five lasers. Cells were sorted as bulk (20 cells/well) and single cell/well into a culture.

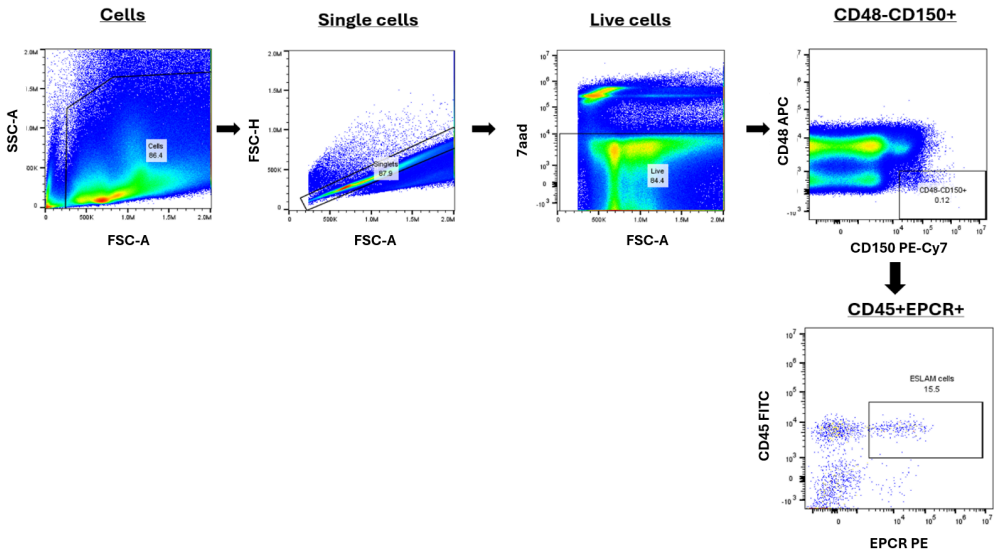


Figure 4: The gating strategy used for FACS isolation of ESLAM cells

The gating strategy used to isolate HSCs from adult mouse BM prior to 10-day cultures. Using forward and side scatter area, cells were gated. Then single cells were gated using forward scatter area and forward scatter height. Live cells were next gated as negative for 7AAD. Then CD150 positive and CD48 negative cells followed by EPCR and CD45 double positive (ESLAM) cells were isolated and sorted. This sort was carried out on the MoFlo.

2.3 Treatment of cells with cytokines

Treatments including IL-4 (Peprotech, 214-14) at a concentration of 1 ng/mL, transforming growth factor-beta (TGF-β) at a concentration of 1 ng/mL, IL-6 (Peprotech, #216-16-10UG) at a concentration of 100 ng/mL and interferon-gamma (IFN-γ) (Biolegend #315-05-20UG) at a concentration of 10 ng/mL were added to the treated wells for each genotype on day 0. The concentrations of IL-4, TGF-β and IFN-γ had been optimised previously by others in the Kent laboratory but IL-6 had not been used so it was important to test a range of concentrations prior to first use (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml). IL-4, IFN-γ and IL-6 are stored in a -20^{o}C and TGF-β is stored in a -80^{o}C freezer and are defrosted post sort briefly to add to the media.

2.4 In vitro HSC expansion protocol

Cells were sorted into cultures containing StemSpan (StemCell, #09650), Fetal Bovine Serum (FBS) (Gibco, 10270-106) at a concentration of 10 µL/mL, L-Glutamine (ThermoFisher, 25030081) used at a concentration of 0.01 µL/mL, penicillin and streptomycin (Sigma-Aldrich, P4333-100mL) used at a concentration of 0.01 µL/mL, □-mercaptoethanol (Gibco, #31350-010) used at a concentration of 0.2 µl/mL, stem cell factor (SCF) (Bio-techne, #455-MC) at a concentration of 300 ng/mL and interleukin (IL)-11 (Bio-techne, #418-ML) at a concentration of 20 ng/mL[(73,247,248)].

Plates were then placed in a sterile and humidified tissue culture 37°C incubator with 5% CO_2 and 20% O_2 for 10 days.

2.5 Images of 10 day cultures

After 10 days in culture, images of the cultures were taken on an Olympus CKX53 microscope at 4x magnification. No alterations were made to any of the images included aside from all being cropped to the same size and a scale bar added.

2.6 Flow cytometric analysis of *in vitro* cultures

After 10 days in this culture the cells were spun down at 350xG for five minutes and resuspended in 100 µL antibody mastermix. Samples were then stained using antibodies as specified in **Table 1** and immunophenotyped on the BD LSRFortessa. Samples were then analysed using the FlowJo v10 (Tree Star) analysis software. The total number of cells was calculated using precision count beads (BioLegend, #424902) as 1ul was added to each sample, the total number present in 1 ul was divided by the total number recorded to provide a factor which was then multiplied by the cell number recorded. The gating strategies involved selecting cells based on the forward and side scatter area, single cells using the forward scatter area and forward scatter height followed by selecting live cells using the 7AAD stain (1:1000). LSK cells were then gated as lineage negative using Ly6G and CD11b as in 10-day ESLAM cultures these tend to be the only lineage markers expressed due to

erythrocyte and lymphocyte development not being favoured [(73)]. This was then followed by c-Kit and Sca-1 positive gates. ELSK cells were next gated as EPCR positive. Lineage positive cells were selected as the populations positive for the selected marker.

Table 1: List of Antibodies used throughout this work

Antibody	Clone	Fluorochrome	Stock supplier	Stock concentration	Dilution
EPCR	1560	PE	eBioscience	0.2 mg/mL	1:100/ 1:1000
CD45	A20	FITC	BioLegend	0.5 mg/mL	1:400
CD48	HM48-1	APC	BioLegend	0.2 mg/mL	1:200
CD150	TC15-12F12.2	PE-Cy7	BioLegend	0.2 mg/mL	1:1000
Sca-1	D7	BV605	BioLegend	0.2 mg/mL	1:100
c-kit	2B8	APC-Cy7	BioLegend	0.2 mg/mL	1:1000
Ly6C	HK1.4	BV605	BioLegend	0.2 mg/mL	1:500
Ly6G	1A8	FITC	BioLegend	0.5 mg/mL	1:500
Sca-1	D7	BV421	BioLegend	0.2 mg/mL	1:500
ESAM	1G8/ESAM	APC	BioLegend	0.2 mg/mL	1:1000
CD11b	M1/70	BV785	BioLegend	0.2 mg/mL	1:500
CD34	RAM34	AF700	eBioscience	0.2 mg/mL	1:500
TER119	TER119	PE-Cy7	eBioscience	0.2 mg/mL	1:500
CD135	A2F10	BV421	BioLegend	0.2 mg/mL	1:500

2.7 Statistical analysis

Comparisons on bar charts were deemed significant through one-way ANOVAs and Welch's ANOVA (when standard deviations were not equal) followed by Dunnett's multiple pairwise comparisons and Fisher's exact test on line graphs using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, www.graphpad.com. Significance is denoted on figures using asterisks (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$).

3.0 Results

3.1 IL-6 increases cell number of wildtype cultures and TET2$^{+/-}$ HSCs are hyporesponsive to IL-4

Previous studies have revealed that inflammatory cytokines can alter HSC survival and expansion in vivo and in vitro, as explained previously [(249)]. We first wanted to assess the impact of IL-6 on wildtype HSCs at a range of concentrations in order to determine the optimum dose for future experiments as the concentration of this cytokine had not been optimised in the Kent laboratory before. We also sought to determine whether wildtype and $TET2^{+/-}$ HSCs respond to the same concentration of IL-4 as previous research had suggested that they may not [(250)].

3.1.1 IL-6 increases total cell number of wild type cultures in a dose-dependent manner

First, we undertook a dose response test to determine the optimum concentration of IL-6 for future analyses. Wildtype ESLAM HSCs were sorted as twenty cells per well and then cultured for 10 days with SCF, IL-11 and different amounts of IL-6. Through these experiments, as shown in **Figure 5**, we showed that when cultured alongside SCF and IL11, addition of IL-6 increases the total number of cells at a concentration of 100 ng/ml ($p=0.0003$) and the total number of ELSKs was also increased at this concentration ($p=0.0593$). We also found that while the percentage of granulocytes was decreased in response to IL-6 ($p=0.0084$ for 0.01 ng/ml, $p=0.0217$ for 0.1 ng/ml, $p=0.0298$ for 1 ng/ml, $p=0.0094$ for 10 ng/ml and $p=0.0091$ for 100 ng/ml), the total number of granulocytes was increased at 100 ng/ml compared with untreated ($p=0.0232$), 0.01 ng/ml ($p=0.0281$), 0.1 ng/ml ($p=0.0255$) and 10 ng/ml ($p=0.003$). As a result, we selected 100 ng/ml as the optimum concentration of IL-6 for future analyses as this concentration had the greatest impact on the ESLAM HSCs.

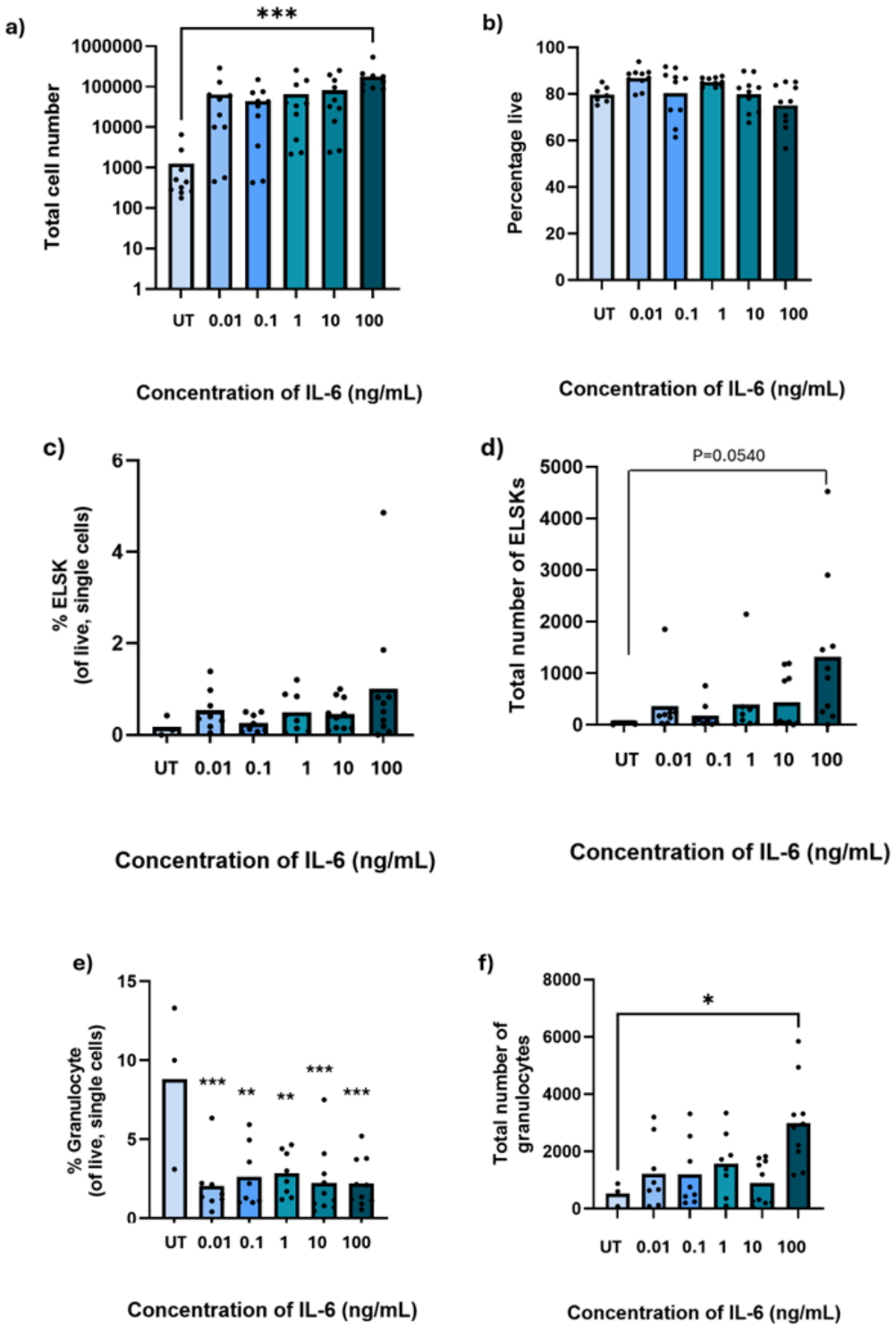

a)
***
Total cell number
1000000
100000
10000
1000
100
10
1
UT 0.01 0.1 1 10 100
Concentration of IL-6 (ng/mL)
b)
Percentage live
100
80
60
40
20
0
UT 0.01 0.1 1 10 100
Concentration of IL-6 (ng/mL)
c)
% ELSK
(of live, single cells)
6
4
2
0
UT 0.01 0.1 1 10 100
Concentration of IL-6 (ng/mL)
d)
P=0.0540
Total number of ELSKs
5000
4000
3000
2000
1000
0
UT 0.01 0.1 1 10 100
Concentration of IL-6 (ng/mL)
e)
% Granulocyte
(of live, single cells)
15
10
5
0
*** ** ** *** ***
UT 0.01 0.1 1 10 100
Concentration of IL-6 (ng/mL)
f)
*
Total number of
granulocytes
8000
6000
4000
2000
0
UT 0.01 0.1 1 10 100
Concentration of IL-6 (ng/mL)

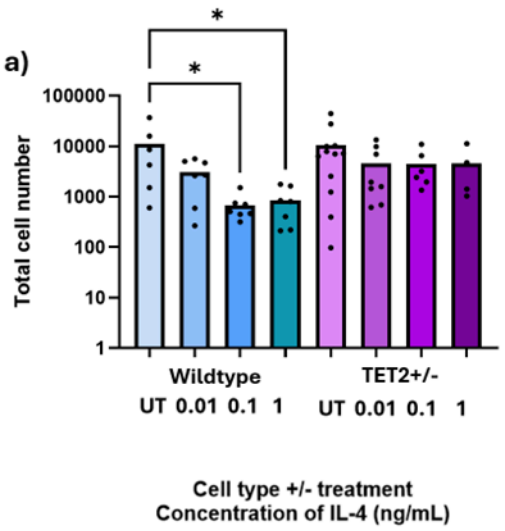
Figure 5: IL-6 increases total cell number of wild-type cultures in a dose dependent manner

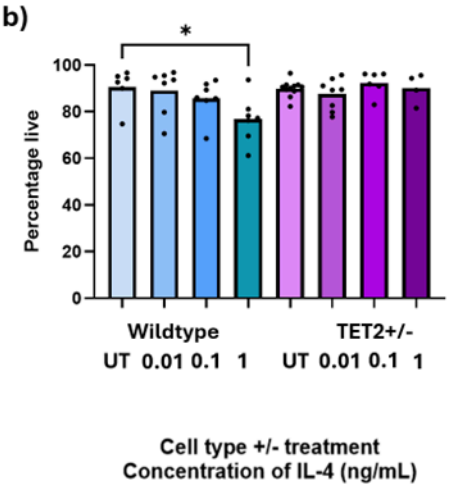
a) The total number of cells per well was calculated and there was a greater number of cells in the cultures treated with 100 ng/mL IL-6 (p=0.0003). **b)** The percentage of live cells, as determined by the addition of 7AAD, demonstrates consistently high viability across all concentrations of IL-6. **c)**There was no change in the percentage of ELSKs at any concentration of IL-6 **d)** but 100 ng/mL does increase the total number of ELSKs compared to untreated (p=0.0540). **e)** IL-6 reduces the percentage of granulocytes (Ly6G and CD11b positive cells) at all concentrations of IL-6 compared with wildtype (from left to right, p=0.0084, p=0.0217, p=0.0298, p=0.0094 and p=0.0091). **f)** IL-6 increases the total number of granulocytes at 100 ng/ml compared with untreated (p=0.0232), 0.01 ng/ml (p=0.0281), 0.1 ng/ml (p=0.0255) and 10 ng/mL (p=0.003). * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=1 [All comparisons displayed on the graph are compared to untreated]

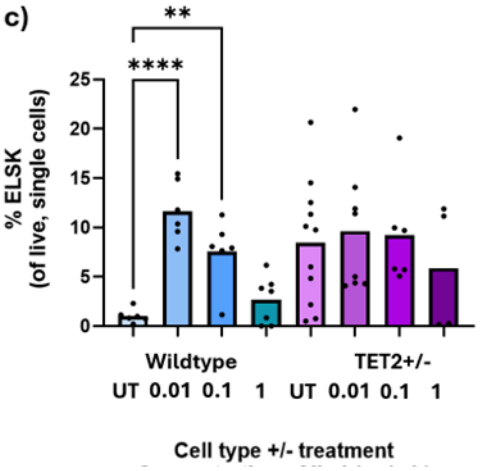
3.1.2 TET2$^{+/-}$ HSCs are hyporesponsive to IL-4

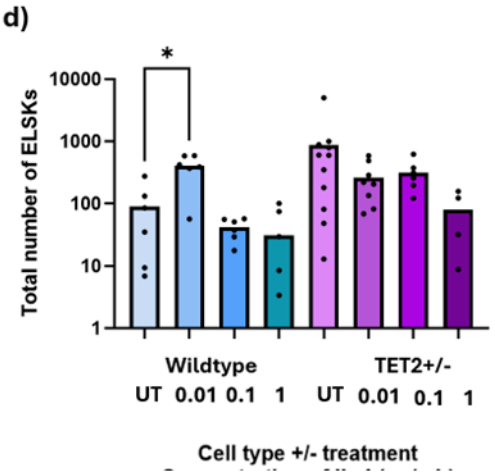
In order to determine whether wildtype and TET2$^{+/-}$ respond to IL-4 at the same concentration, ESLAM HSCs were isolated by FACS and cultured for 10 days with the addition of IL-4 at the concentrations of 0.01, 0.1 and 1 ng/ml. By investigating the concentration of IL-4 at which wildtype and TET2$^{+/-}$ ESLAM HSCs respond, we showed that TET2$^{+/-}$ HSCs were less sensitive to low concentrations of IL-4 than wildtype HSCs, as shown in **Figure 6**. We found that wildtype cultures had a reduced percentage of live cells after 10 days at 1 ng/ml of IL-4 (p=0.0129) whereas TET2$^{+/-}$ cultures did not have reduced survival at any concentration of IL-4. Flow cytometry endpoint analyses also showed that TET2$^{+/-}$ untreated cultures retained a greater total number of primitive ELSKs compared with wildtype (p=0.0256) after 10 days although total number of ELSK cells in TET2$^{+/-}$ cultures decreases back down to

a level comparable to wildtype untreated (p=>0.9999) at 1 ng/ml of IL-4. The ability of single wildtype HSCs to form colonies decreased from a concentration of 0.01 ng/ml of IL-4 whereas IL-4 did not prevent single $TET2^{+/-}$ HSCs from forming colonies but was observed to reduce the size of colonies formed beyond 0.1 ng/ml as determined using ImageJ to measure the diameter of colonies and the bead counts from the colonies analysed.


a)
*
*
Total cell number
100000
10000
1000
100
10
1
Wildtype
UT 0.01 0.1 1
TET2+/-
UT 0.01 0.1 1
Cell type +/- treatment
Concentration of IL-4 (ng/mL)


b)
*
Percentage live
100
80
60
40
20
0
Wildtype
UT 0.01 0.1 1
TET2+/-
UT 0.01 0.1 1
Cell type +/- treatment
Concentration of IL-4 (ng/mL)


c)
**
****
% ELSK
(of live, single cells)
25
20
15
10
5
0
Wildtype
UT 0.01 0.1 1
TET2+/-
UT 0.01 0.1 1
Cell type +/- treatment


d)
*
Total number of ELSKs
10000
1000
100
10
1
Wildtype
UT 0.01 0.1 1
TET2+/-
UT 0.01 0.1 1
Cell type +/- treatment

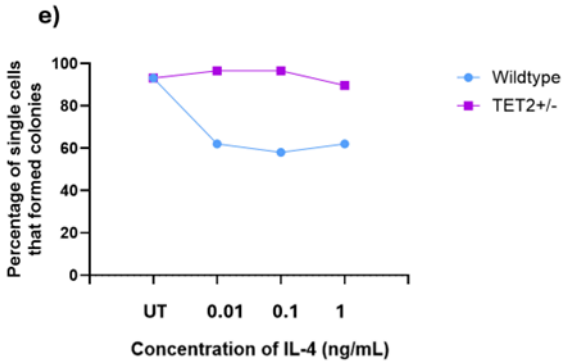

e)
Percentage of single cells
that formed colonies
100
80
60
40
20
0
UT
0.01
0.1
1
Concentration of IL-4 (ng/mL)
Wildtype
TET2+/-

Figure 6: TET2$^{+/-}$ HSCs are hyporesponsive to IL-4

a) IL-4 decreases total cell number for wildtype cultures at 0.1 ng/mL (p=0.0349) and 1 ng/mL (p=0.0386) and there is a modest decrease in cell number for TET2+/- cultures at 0.01 ng/mL (p=0.3871), 0.1 ng/mL (p=0.4407) and (p=0.5839) **b)** At 1 ng/ml, IL-4 results in a reduced percentage live for wildtype cultures (p=0.0129) as determined by the addition of 7AAD. **c)** In wildtype cultures, the percentage of ELSK cells increases at 0.01 ng/ml (p=0.0099) but this decreases back to untreated levels at 1 ng/ml of IL-4 (p=0.0345). **d)** TET2$^{+/-}$ untreated cultures retain a greater total number of ELSKs compared with wildtype (p=0.0256) and at 1 ng/ml wildtype cultures have a reduced total number of ELSKs compared with at 0.01 ng/ml (p=0.0424). The total number of ELSK cells in TET2$^{+/-}$ cultures decreases back down to a level comparable to wildtype untreated (p=>0.9999) at 1 ng/ml IL-4 **e)** The ability of single wildtype HSCs to form colonies is reduced from 0.01 ng/ml of IL-4 but there is no reduction for single TET2$^{+/-}$ HSCs. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=1

3.2 IL-4 and IFN-γ decrease total cell number of TET-2^{+-} cultures while IL-6 increases total cell number

In order to assess the proliferation, G0 exit and early divisional kinetics of ESLAM HSCs in response to different cytokines, we cultured cells for 10 days with the addition of cytokines to the treated wells. We counted cells on days 1-3 after sorting and calculated the percentage of cells that had undergone the first and second division for each genotype (both treated and untreated). After 10 days in culture, we added precision count beads to each well and calculated the number of cells using the count beads as a reference as described previously [(35,251)]. We also calculated the colony areas using ImageJ to provide a quantitative measure for colony morphology.

3.2.1 Wildtype and TET2$^{+/-}$ cultures exhibit colony spreading in response to IL-4

As shown in **Figure 7a**, we found that both wildtype and TET2$^{+/-}$ cultures display colony spreading in response to IL-4 and move away from the centre of the well. We found that IFN-γ reduced the size of the colony without promoting migration away from the centre of the well (**Figure 7b**). TGF-β did not have consistent effects on colony size or density (**Figure 7c**) but IL-6 increased the size of colonies but does not appear to affect colony morphology (**Figure 7d**).

As a result of certain noticeable differences in the colony morphology in response to each cytokine treatment, we decided to determine whether there were significant differences in the colony area, shown in **Figure 8**. This was achieved using ImageJ

with a scale set to calculate the area of each colony using images taken at the same magnification. The area of the colony produced by HSCs was reduced by IL-4 for both wildtype (p=<0.0001) and $TET2^{+/-}$ (p=<0.0001). We found that the area of colonies was reduced in response to IFN-γ for both wildtype (p=<0.0001) and $TET2^{+/-}$ (p=<0.0001). There was a moderate reduction in response to TGF-□ for wildtype (p=0.0192) and $TET2^{+/-}$ (p=0.0041). In response to IL-6, there was also moderate reduction in the area of colonies for both wildtype (p=<0.0001) and $TET2^{+/-}$ (p=0.0239).

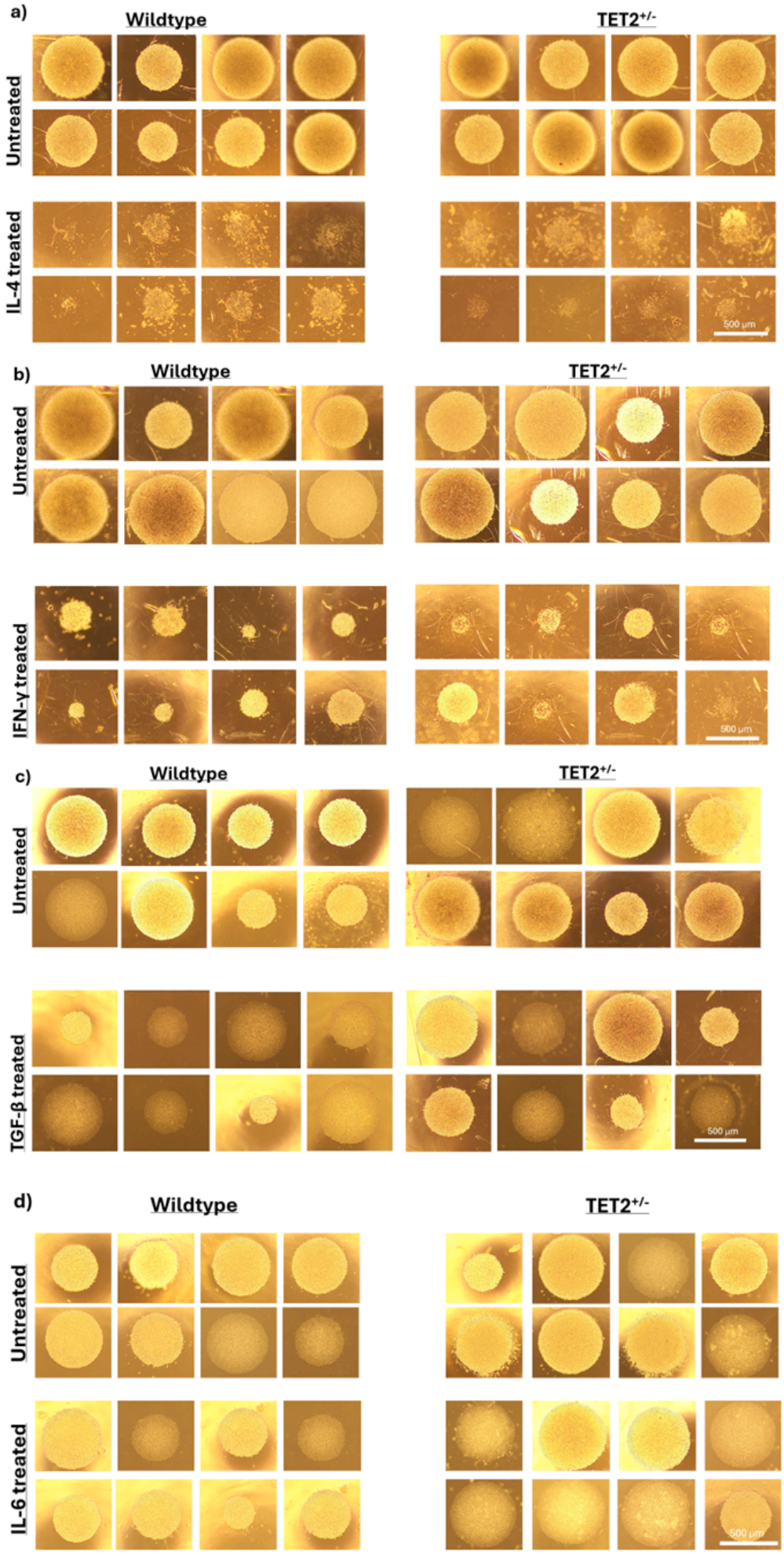

a)
Wildtype
TET2+/-
Untreated
IL-4 treated
500 µm
b)
Wildtype
TET2+/-
Untreated
IFN-γ treated
500 µm
c)
Wildtype
TET2+/-
Untreated
TGF-β treated
500 µm
d)
Wildtype
TET2+/-
Untreated
IL-6 treated
500 µm

Figure 7: IL-4 encourages colony spreading of TET2$^{+/-}$ cultures

a) IL-4 reduces the size of colonies and encourages colony spreading away from the centre of the wells promoting the elongated phenotype of the cells. **b)** IFN-Y reduces the size of colonies and does not promote colony spreading. **c)** TGF-β appears to have less consistent effects on the cultures with no obvious changes in colony size or density. **d)** IL-6 increases the size and density of cultures of both genotypes. A 500 micrometre scale bar is included on each for reference. N=3 per treatment (12)

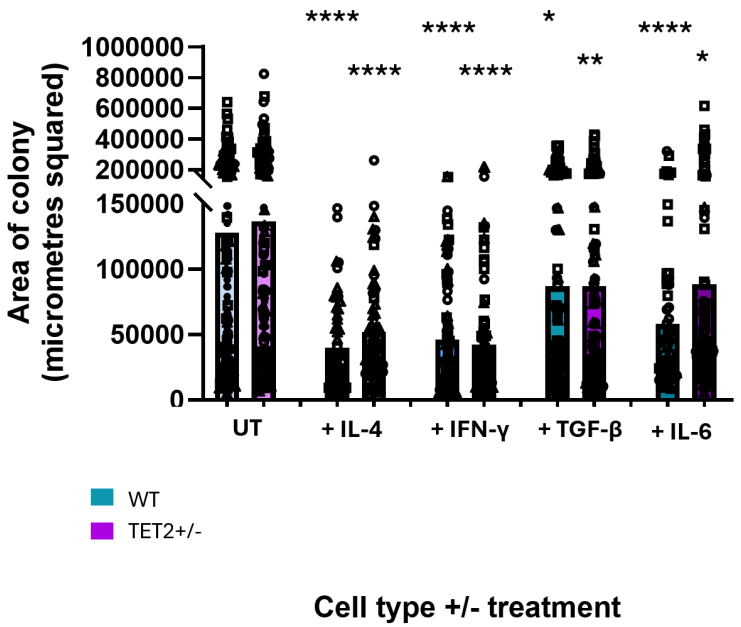


Figure 8: Cytokine treatments reduce the area of colonies produced by wildtype and TET2$^{+/-}$ HSCs

The area of each colony was calculated using ImageJ. The area of the colony produced by HSCs was reduced by IL-4 for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001). The area of colonies was also reduced in response to IFN-γ for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001). There was a moderate reduction in response to TGF-□ for wildtype (p=0.0192) and TET2$^{+/-}$ (p=0.0041). In response to IL-6, there was a moderate reduction in the area of colonies for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=0.0239). Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

3.2.2 IL-4 and IFN-γ treatment leads to decrease clone size

Through calculating the total cell number, we showed that the total cell number is reduced in response to IL-4 for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) cultures, as shown in **Figure 9**. The total cell number is also reduced in response to IFN-γ for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) cultures. There is no significant difference in the total number of cells in response to TGF-β for wildtype (p=0.3099) or TET2$^{+/-}$ (p=0.4351) cultures. We also showed that treatment with IL-6 increases the total cell number for both wildtype (p=0.0027) and TET2$^{+/-}$ (p=<0.0001). We also confirmed that there was no significant difference in total cell number between cultures of wildtype and TET2$^{+/-}$ cells (p=>0.9999).

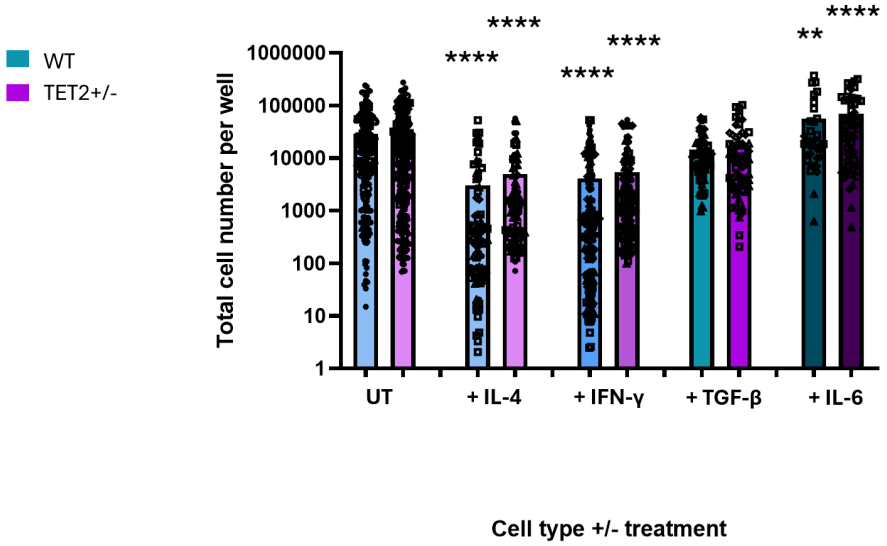


Figure 9: Total cell number is reduced for both genotypes by IFN-γ and IL-4 but increased by IL-6

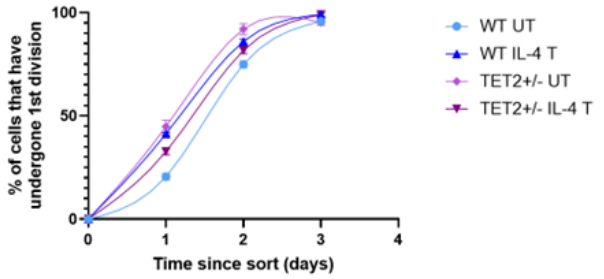
The total cell number is reduced in response to IL-4 for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) cultures. The total cell number is also reduced in response to IFN-γ for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) cultures. There is no change in cell number in response to TGF-β for wildtype (p=0.3099) or TET2$^{+/-}$ (p=0.4351) cultures. IL-6 results in an increase in the total number of cells for both wildtype (p=0.0027) and TET2$^{+/-}$ (p=<0.0001) cultures. Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by

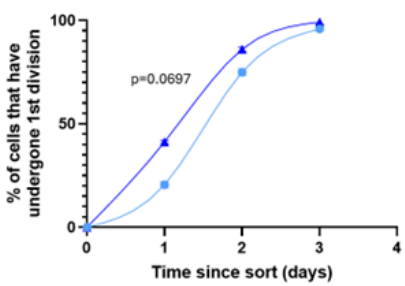
differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

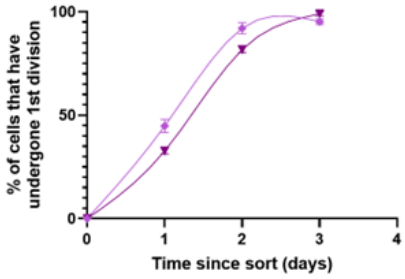
3.2.3 TET2$^{+/-}$ HSCs exhibits slower cell cycle kinetics in response to cytokines

Single culture wells initiated with single ESLAM HSCs were counted on days one, two and three to assess the divisional kinetics of wildtype and TET2$^{+/-}$ HSCs untreated and in response to the cytokine treatments. As shown in **Figure 10**, we found that wildtype treated HSCs divide faster than untreated in response to IFN-γ (p=0.0171), TGF-β (p=0.0005) and IL-6 (p=0.0199). There is also a modest increase for wildtype cultures treated with IL-4 compared to untreated (p=0.0697). However, TET2$^{+/-}$ treated HSCs do not divide faster compared to untreated in response to IL-4 (p=0.2776), IFN-γ (p=0.1178), TGF-□ (p=0.1494) or IL-6 (p=0.3356). We found no significant differences in the divisional kinetics of wildtype untreated and TET2$^{+/-}$ untreated (p=0.4154) [shown in **Figure 23** in the appendix].

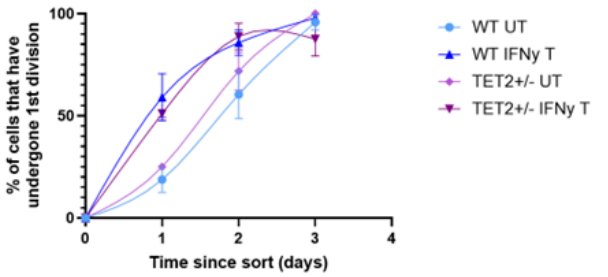
a)

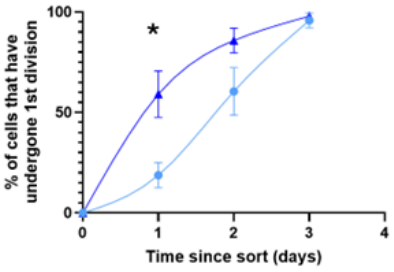

% of cells that have undergone 1st division
Time since sort (days)
WT UT
WT IL-4 T
TET2+/- UT
TET2+/- IL-4 T

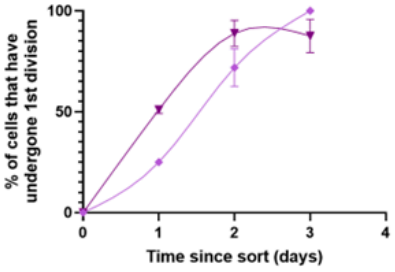

% of cells that have undergone 1st division
p=0.0697
Time since sort (days)
WT UT
WT IL-4 T


% of cells that have undergone 1st division
Time since sort (days)
TET2+/- UT
TET2+/- IL-4 T

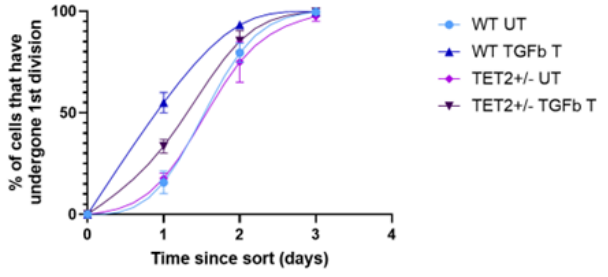
b)

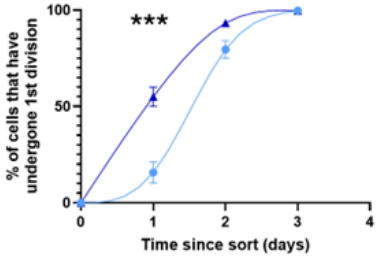

% of cells that have undergone 1st division
Time since sort (days)
WT UT
WT IFNy T
TET2+/- UT
TET2+/- IFNy T

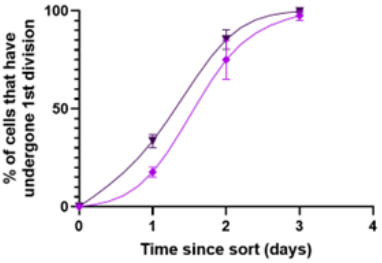

% of cells that have undergone 1st division
*
Time since sort (days)
WT UT
WT IFNy T


% of cells that have undergone 1st division
Time since sort (days)
TET2+/- UT
TET2+/- IFNy T

c)


% of cells that have undergone 1st division
Time since sort (days)
WT UT
WT TGFb T
TET2+/- UT
TET2+/- TGFb T


% of cells that have undergone 1st division
***
Time since sort (days)
WT UT
WT TGFb T


% of cells that have undergone 1st division
Time since sort (days)
TET2+/- UT
TET2+/- TGFb T

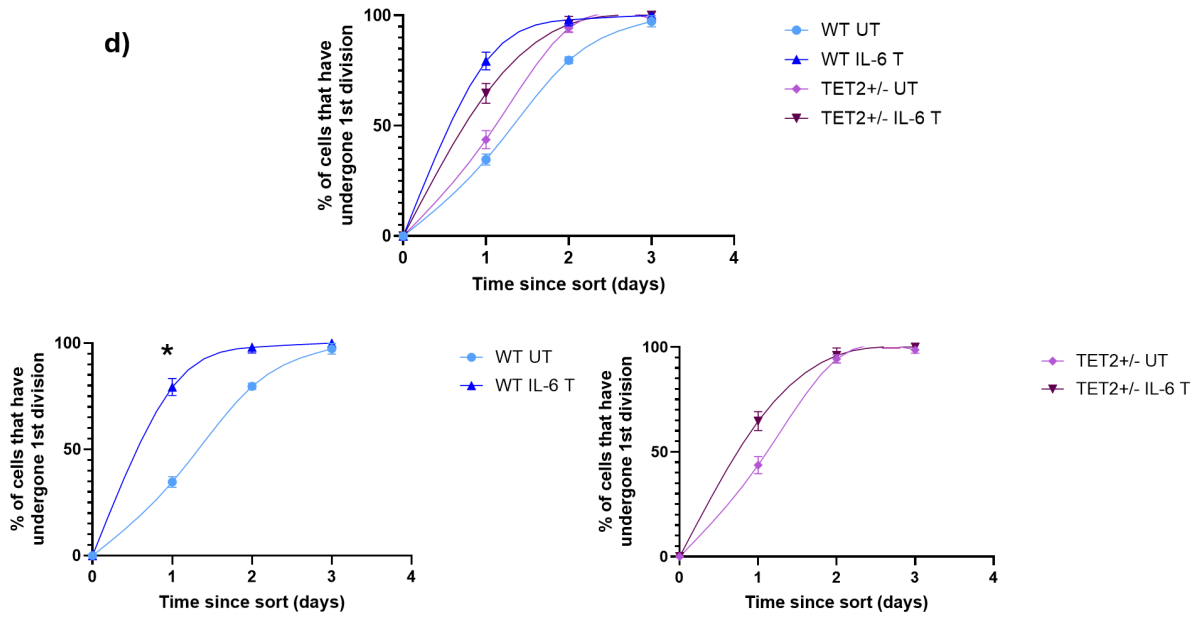


Figure 10: TET2$^{+/-}$ HSCs do not divide faster in response to cytokines

The percentage of cells that had undergone the first division on days 1-3 since the sort was calculated for each genotype both treated and untreated. Significance was calculated using Fisher's exact test. Wildtype HSCs divide more rapidly than untreated in response to **a)** IL-4 (p=0.0697), **b)** IFN-γ (p=0.0171), **c)** TGF-β (p=0.0005) and **d)** IL-6 (p=0.0199) but TET2$^{+/-}$ HSCs do not divide faster than untreated in response to any of the cytokines (p=0.2776, p=0.1178, p=0.1494, p=0.3356, respectively). It also appears that TET2$^{+/-}$ cultures are the first to experience death in response to IFN-γ on day 3. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12) [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

3.3 IL-6 and TGF-β promote differentiation of TET2$^{+/-}$ HSCs and TGF-β reduces the proportion of myeloid lineage cells within TET2$^{+/-}$ cultures

With the aim of adding to recent data showing the differing cell fate of wildtype and TET2$^{+/-}$ HSCs, FACS was used to isolate HSCs from mouse bones, and these cells were then cultured for 10 days with or without the addition of cytokines. After 10 days in culture, the cultures were then analysed using flow cytometry with the gating strategies shown in **Figure 11**. This gating strategy uses ELSK as the preferred

denomination of HSCs. The addition of EPCR to the LSK gating strategy allows for further refinement of this population to enrich for truly functional HSCs [(252)].

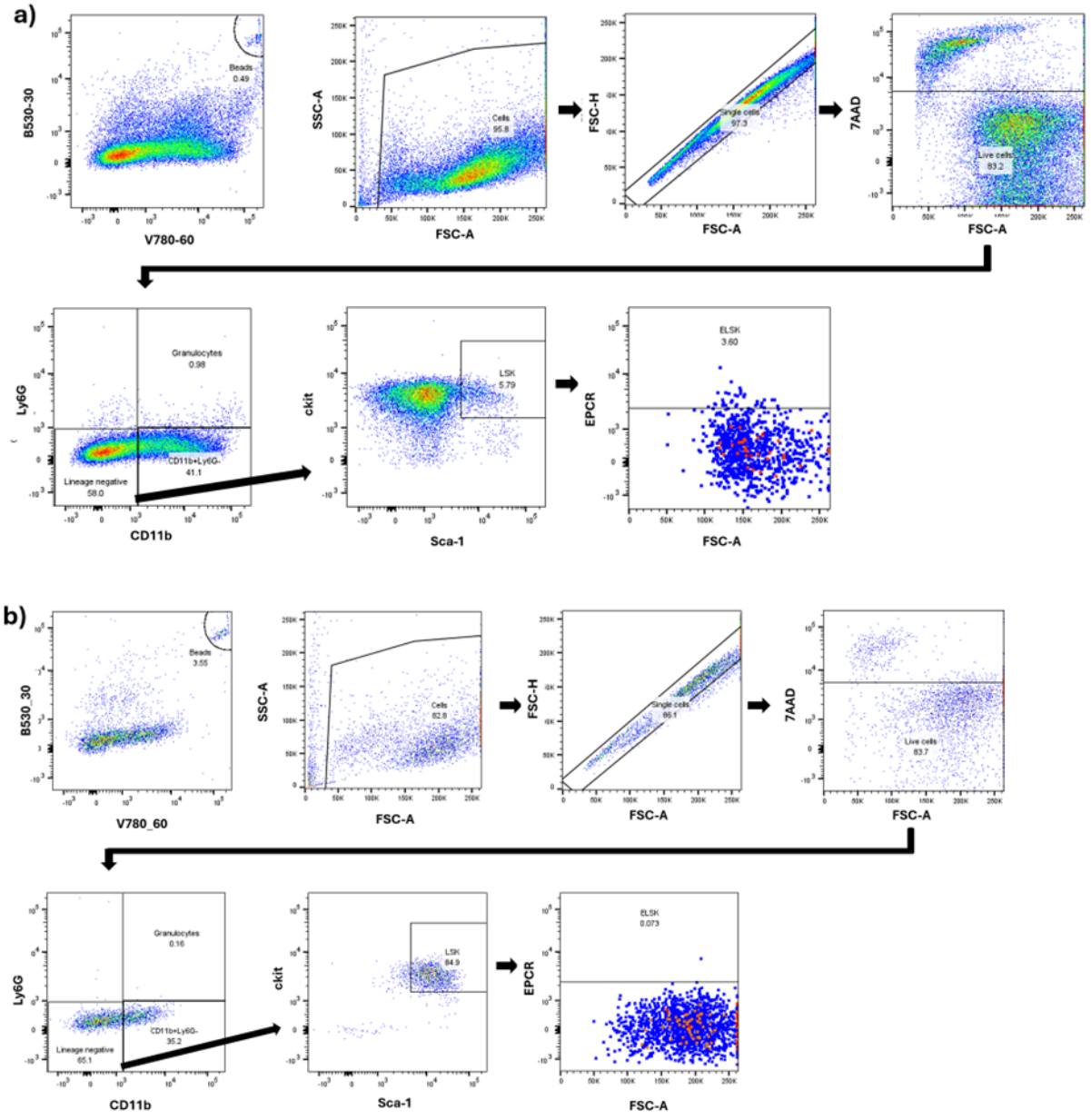


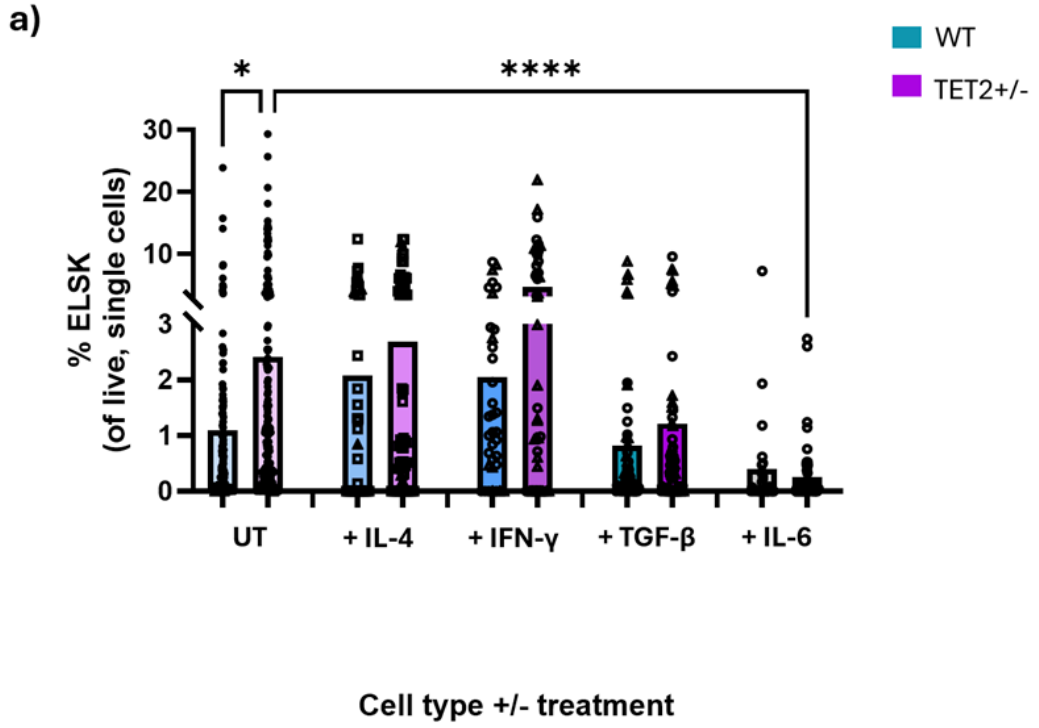
Figure 11: The gating strategies used to isolate and quantify ELSKs, granulocytes and CD11b^{+}Ly6G^{-} cells from cultures

First beads were selected as high fluorescence in the B530_30 and V780_60 channels. Then cells using forward scatter area and side scatter area and next single cells using forward scatter area and height. Then live cells were selected as 7AAD negative. Next cells negative for both Ly6G and CD11b were selected as lineage negative cells, cells positive for CD11b but negative for Ly6G were also selected and cells positive for both of these markers were selected as granulocytes. Then, following on from lineage negative, cells positive for both cKit and Sca-1 were selected as LSKs and finally cells positive for EPCR as well were selected and termed ELSK. **a)** shows this strategy for wildtype untreated cells and **b)** shows the same for IFN-γ treated wildtype cells.

3.3.1 IL-6 and TGF-β promote differentiation of TET2$^{+/-}$ HSCs

Through the analyses of the 10 day cultures, using the gating strategies explained in **Figure 11** and **Figure 13**, we found that untreated TET2$^{+/-}$ HSCs retain a greater percentage of ELSKs in culture than wildtype untreated (p=0.0404), as shown in **Figure 12.** TET2$^{+/-}$ untreated HSCs also retain a greater percentage of ESAM and EPCR double-positive cells than wildtype untreated after 10 days in culture (p=0.0298), as shown in **Figure 14**. There are no significant changes in the percentage of ELSKs for wildtype in response to IL-4, IFN-Y, TGF-β or IL-6 (p=0.7779, p=0.7616, p=>0.9999 and p=0.6634, respectively). However, TET2$^{+/-}$ cultures have a reduced percentage of ELSKs in response to IL-6 (p=<0.0001). This reduction is not observed in response to IL-4, IFN-Y or TGF-β (p=>0.9999, p=0.4594 and p=0.2443 respectively). The percentage of ESAM and EPCR double positive cells is also reduced for both wildtype and TET2$^{+/-}$ cultures in response to TGF-β (p=0.0036 and p=0.0031, respectively). The percentage of these cells is also reduced in response to IL-6 for both wildtype (p=0.0032) and TET2$^{+/-}$ (p=<0.0001). TET2$^{+/-}$ cultures retain a greater total number of ELSKs than wildtype (p=0.0143) and a greater total number of ESAM and EPCR double positive cells than wildtype (p=.0.0298) IL-4 results in a decrease in the total number of ELSKs for wildtype cultures (p=0.0273) but not for TET2$^{+/-}$ cultures (p=0.9998) and the same is true for ESAM and EPCR double positive cells (p=0.0085 and p=0.1981, respectively). TGF-β results in a decrease in the total number of ELSKs for TET2$^{+/-}$ (p=0.0006) but not for wildtype (p=0.2936). However, TGF-β decreases the total number of ESAM+EPCR+ cells for both wildtype (p=0.0091) and TET2$^{+/-}$ cultures (p=0.0001). A decrease in the total number of ELSKs is also observed for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) in response to IL-6. The total number of ESAM+EPCR+ cells is decreased for only TET2$^{+/-}$ cultures in response to IL-6 (p=<0.0001).

a)


WT
TET2+/-
*
****
% ELSK
(of live, single cells)
30
20
10
3
2
1
0
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
Cell type +/- treatment

b)

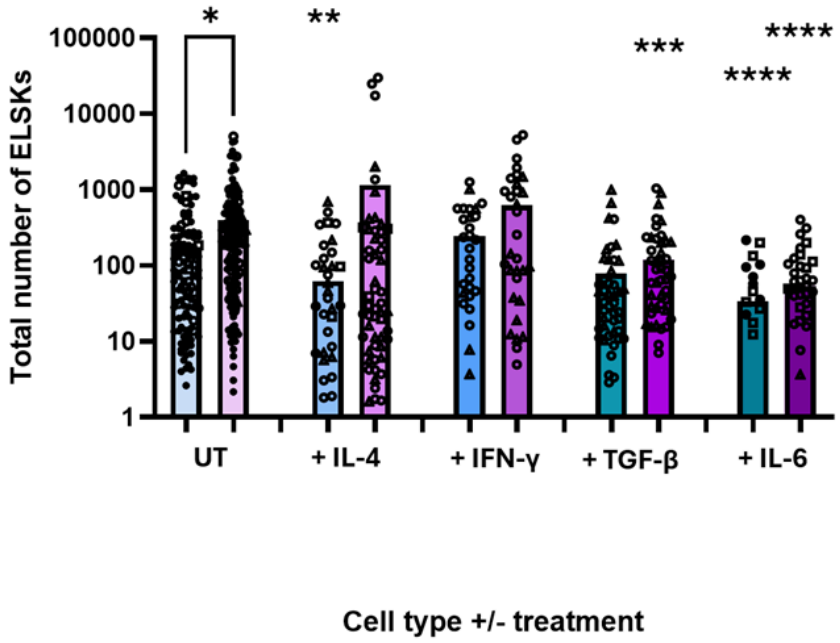

*
**
***
****
****
Total number of ELSKs
100000
10000
1000
100
10
1
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
Cell type +/- treatment

Figure 12: IL-6 and TGF-β promote differentiation of TET2$^{+/-}$ HSCs

a) After 10 days, TET2$^{+/-}$ HSCs retain a greater percentage of ELSKs in culture than wildtype (p=0.0404). There are no significant changes in the percentage of ELSKs for wildtype in response to IL-4, IFN-Y, TGF-β or IL-6 (p=0.3124, p=0.3063, p=0.9913 and p=0.2292, respectively). However, TET2$^{+/-}$ cultures have a reduced percentage of ELSKs in response to IL-6 (p=<0.0001). This reduction is not observed in response to IL-4, IFN-Y or TGF-β (p=>0.9999, p=0.1401 and p=0.0615 respectively). **b)** TET2$^{+/-}$ cultures retain a greater total number of ELSKs than wildtype (p=0.0143). IL-4 results in a decrease in the total number of ELSKs for wildtype cultures (p=0.0062) but not for TET2$^{+/-}$ cultures (p=0.8927). TGF-β results in a decrease in the total number of ELSKs for TET2$^{+/-}$ (p=0.0001) but not for wildtype (p=0.0760). A decrease in the total number of ELSKs is observed for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) in response to IL-6. Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

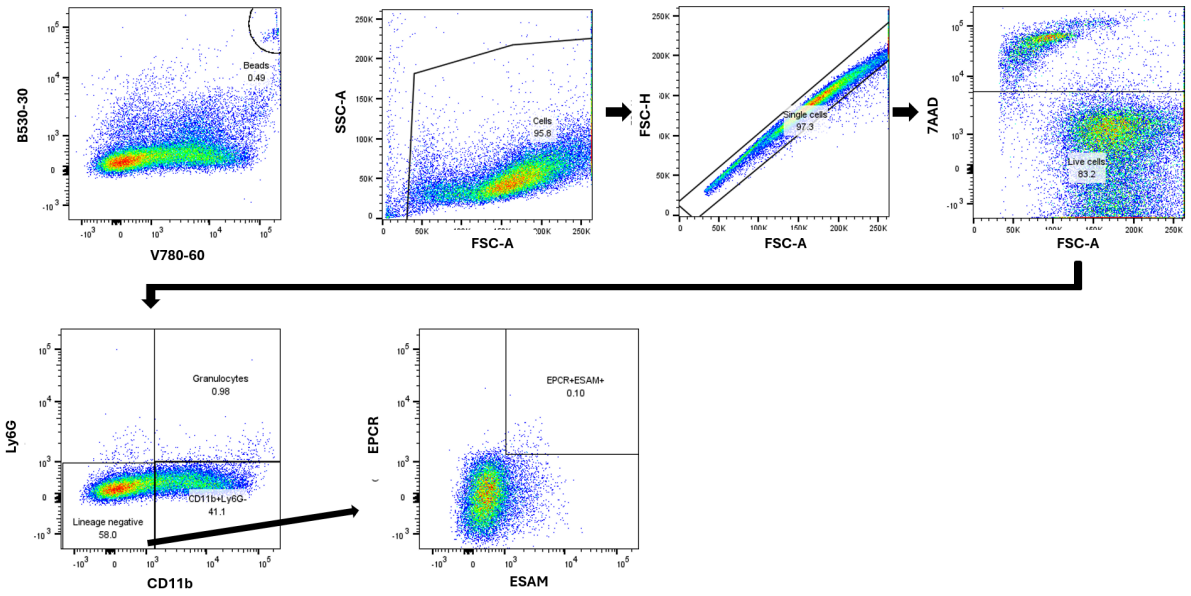
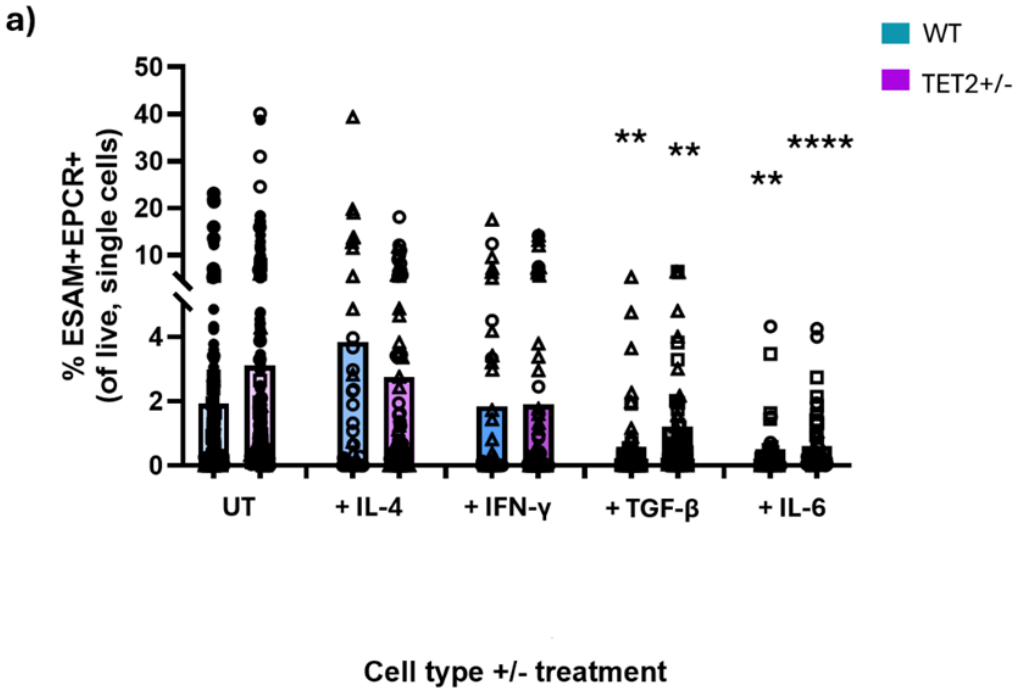


Figure 13: The gating strategy used to isolate and quantify EPCR^{+}ESAM^{+} cells

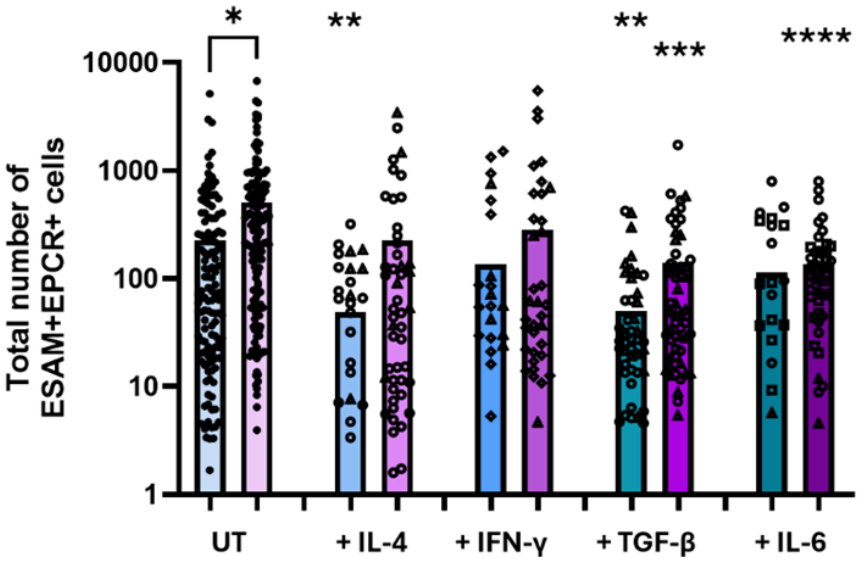
First beads were selected as high fluorescence in the B530_30 and V780_60 channels. Then cells using forward scatter area and side scatter area and next single cells using forward scatter area and height. Then live cells were selected as 7AAD negative. Next cells negative for both Ly6G and CD11b were selected as lineage negative cells, cells positive for CD11b but negative for Ly6G were also selected and cells positive for both of these markers were selected as granulocytes. Then, following on from lineage negative, cells positive for both EPCR and ESAM were isolated and quantified.

a)



Cell type +/- treatment

b)

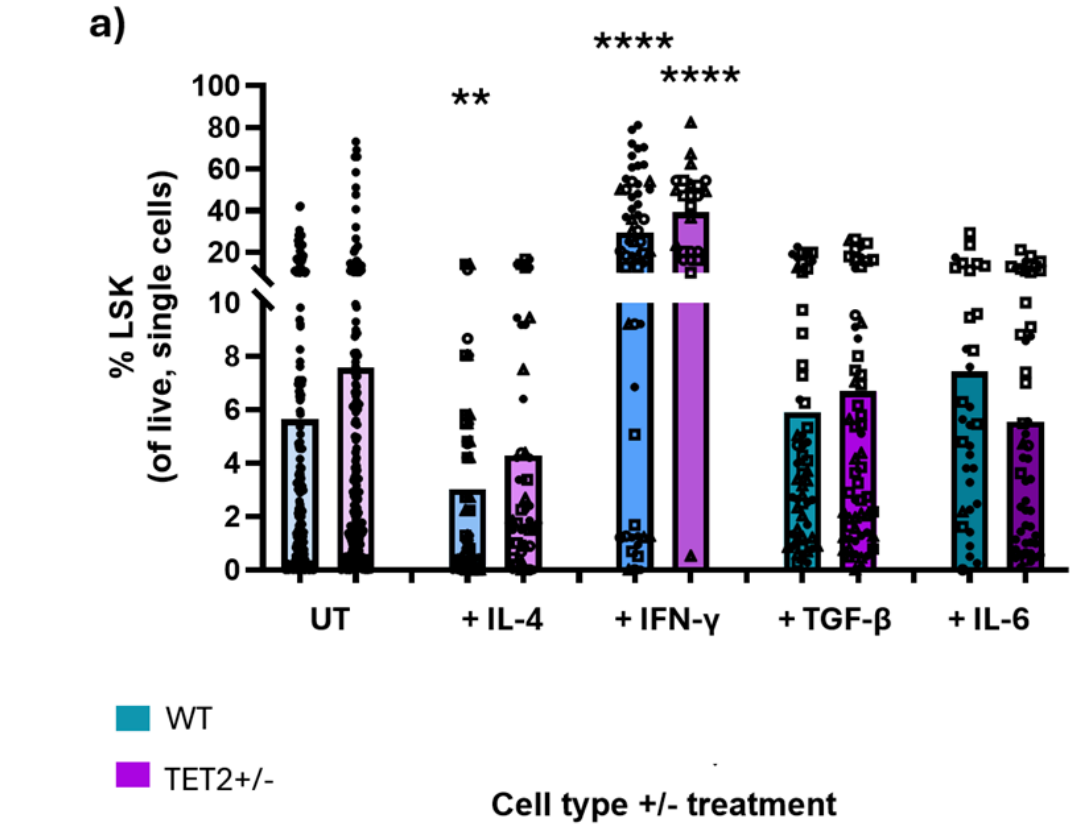


Cell type +/- treatment

Figure 14: IL-6 and TGF-□ reduce the total number of $TET2^{+/-}$ $ESAM^{+}EPCR^{+}$ cells

a) The percentage of ESAM and EPCR double positive cells is reduced for both wildtype and $TET2^{+/-}$ cultures in response to TGF-β (p=0.0036 and p=0.0031, respectively). The percentage of the same cells is also reduced in response to IL-6 for both wildtype (p=0.0032) and $TET2^{+/-}$ (p=<0.0001). **b)** $TET2^{+/-}$ untreated cultures retain a greater total number of $ESAM^{+}EPCR^{+}$ cells after 10 days than wildtype untreated cultures (p=0.0298). IL-4 results in a reduced number of these cells for wildtype cultures (p=0.0085) but not for $TET2^{+/-}$ (p=0.1981). TGF-β decreases the total number of $ESAM^{+}EPCR^{+}$ cells for both wildtype (p=0.0091) and $TET2^{+/-}$ cultures (p=0.0001). The total number of these cells is also decreased for $TET2^{+/-}$ cultures in response to IL-6 (p=<0.0001). Wildtype is shown in blue and $TET2^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

As the gating for ELSKs has not yet been validated for the 10-day culture system, we also collected the LSK data for all experiments, as shown in **Figure 15**. As sca-1 is interferon-inducible, the percentage of and total number of LSKs increases in IFN-γ treated cultures for wildtype (p=<0.0001 and p=0.0317, respectively). IFN-γ also increases the percentage of LSKs for $TET2^{+/-}$ cultures (p=<0.0001) but the total number of LSKs is not increased (p=0.1022). This is most likely because the number of LSKs is slightly higher in $TET2^{+/-}$ untreated cultures compared to wildtype untreated cultures (p=0.0234). We demonstrate that IL-4 reduces the percentage of LSKs for wildtype cultures (p=0.0036) but not for $TET2^{+/-}$ cultures (p=0.0719) and the total number of LSKs in wildtype cultures (p=<0.0001). We also showed that TGF-β decreased the total number of LSKs in $TET2^{+/-}$ cultures (p=0.0010) but not in wildtype cultures (p=0.2387).


a)
% LSK
(of live, single cells)
100
80
60
40
20
10
8
6
4
2
0
**
****
****
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
WT
TET2+/-
Cell type +/- treatment

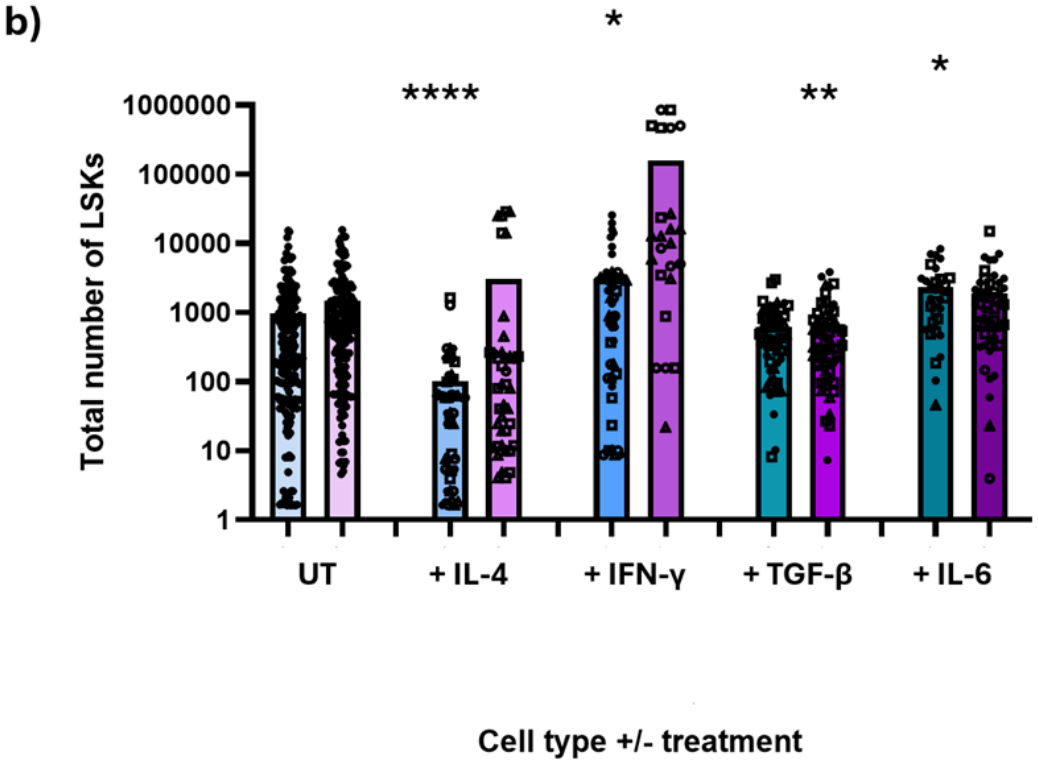

b)
Total number of LSKs
1000000
100000
10000
1000
100
10
1
****
*
**
*
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
Cell type +/- treatment

Figure 15: IL-4 does not impact the percentage or total number of LSKs in TET2$^{+/-}$ cultures

a) IL-4 reduces the percentage of LSKs for wildtype cultures (p=0.0036) but not for TET2$^{+/-}$ cultures (p=0.0719). IFN-γ increases the percentage of LSKs for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001). **b)** IL-4 also reduces the total number of LSKs in wildtype cultures (p=<0.0001). IFN-γ increases the total number of LSKs in wildtype cultures (p=0.0317) as does IL-6 (p=0.0238). TGF-β decreased the total number of LSKs in TET2$^{+/-}$ cultures (p=0.0010). Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

3.3.2 TET2$^{+/-}$ HSCs produce fewer granulocytes and CD11^{+}Ly6G^{-} in response to TGF-β

Granulocytes (defined as CD11b and Ly6G double-positive) are shown in the gating strategy in **Figure 11**. TET2$^{+/-}$ HSCs produce a greater percentage of myeloid lineage cells than wildtype HSCs (p=0.0216), as shown in **Figure 16**. After treatment with IFN-γ, TET2$^{+/-}$ HSCs produce a greater percentage of myeloid lineage cells than wildtype treated HSCs do (p=0.0062). TGF-β results in a decrease in the percentage of myeloid lineage cells produced by TET2$^{+/-}$ HSCs (p=0.0004) and for wildtype (p=0.0555). TET2$^{+/-}$ untreated HSCs also produce a greater number of myeloid lineage cells in culture than wildtype untreated HSCs (p=0.0142). IL-4, IFN-γ and TGF-β all result in a decrease in the total number of myeloid lineage cells for both wildtype (p=0.0211, p=0.0491 and p=0.0026, respectively) and TET2$^{+/-}$ (p=0.0003, p=0.0014 and p=<0.0001, respectively). There is no change in the number of these cells in response to IL-6 for either wildtype (p=0.9942) or TET2$^{+/-}$ cultures (p=>0.9999).

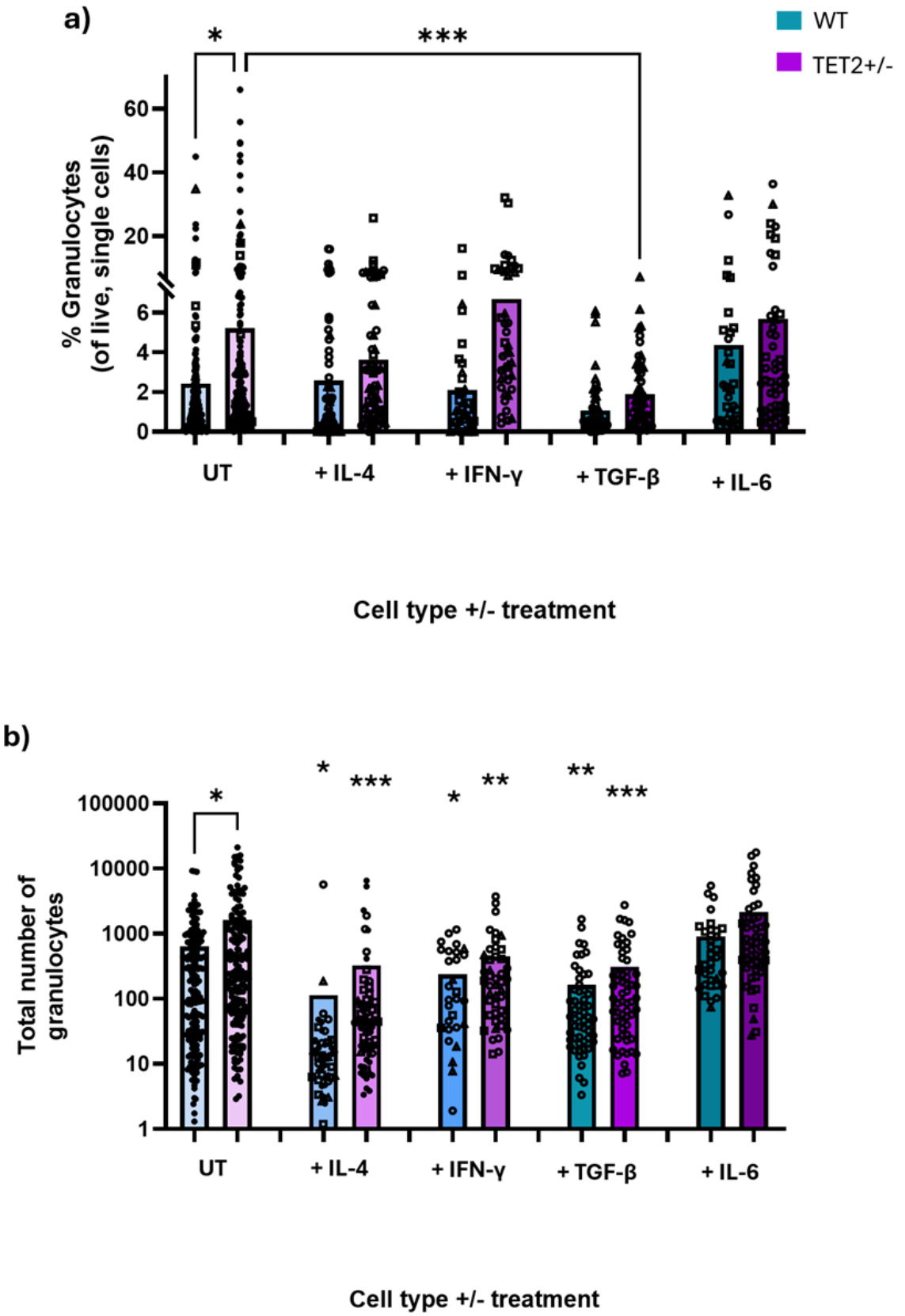
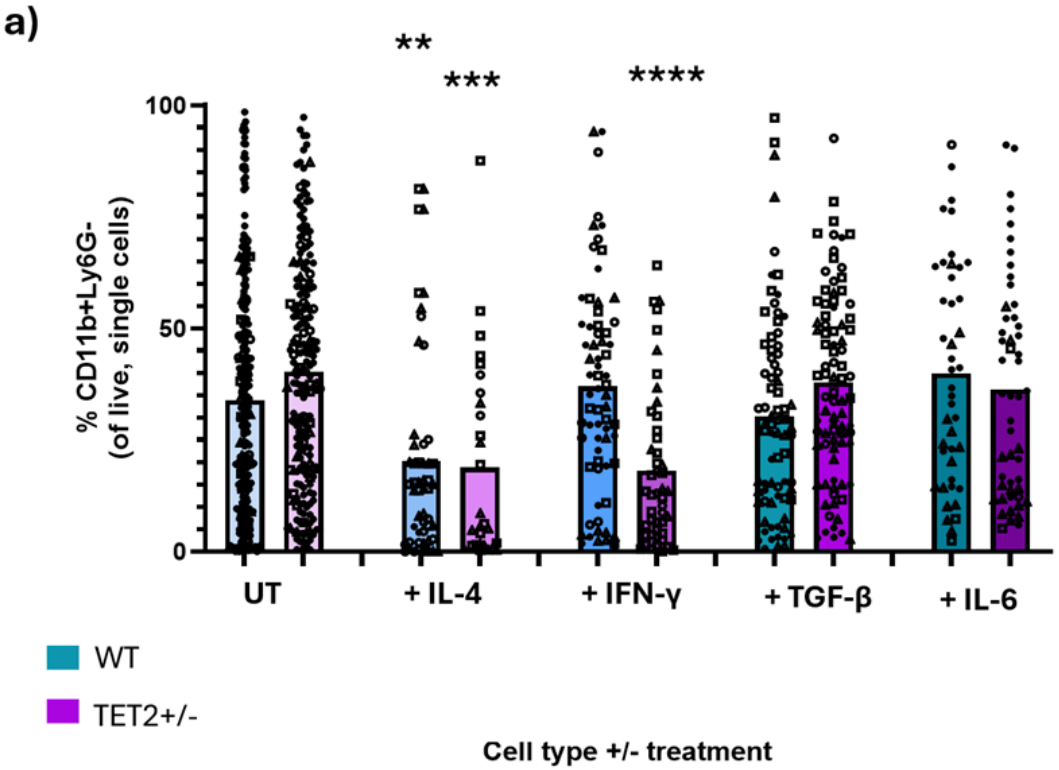

a)
WT
TET2+/-
*
***
% Granulocytes (of live, single cells)
60
40
20
6
4
2
0
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
Cell type +/- treatment
b)
*
*
***
*
**
**
***
Total number of granulocytes
100000
10000
1000
100
10
1
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
Cell type +/- treatment

Figure 16: TET2$^{+/-}$ HSCs produce fewer granulocytes in response to TGF-β

Granulocytes are defined as CD11b and Ly6G double-positive and **a)**TET2$^{+/-}$ HSCs produce a greater percentage of granulocytes than wildtype (p=0.0216). After treatment with IFN-γ, TET2$^{+/-}$ HSCs produce a greater percentage of granulocytes than wildtype treated HSCs do (p=0.0062). TGF-β results in a decrease in the percentage of granulocytes produced by TET2$^{+/-}$ HSCs (p=0.0004) and wildtype (p=0.0555). **b)** TET2$^{+/-}$ HSCs produce a greater number of granulocytes in culture than wildtype (p=0.0142). IL-4, IFN-γ and TGF-β all result in a decrease in the total number of granulocytes for both wildtype (p=0.0211, p=0.0491, p=0.0026, respectively) and TET2$^{+/-}$ (p=0.0003, p=0.0014, p=<0.0001, respectively). Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

IL-4 reduces the percentage of CD11b^{+}Ly6G^{-} cells for both wildtype and TET2$^{+/-}$ cultures (p=0.0059 and p=0.0004, respectively), as shown in **Figure 17**. Despite this, only wildtype ESLAM HSCs produce a reduced total number of CD11b^{+}Ly6G^{-} cells in response to IL-4 (p=0.0021). IL-4-treated TET2$^{+/-}$ ESLAM HSCs also produce a greater total number of CD11b^{+}Ly6G^{-} cells compared to wildtype IL-4 treated (p=0.003). In response to IFN-γ, TET2$^{+/-}$ cultures have a reduced percentage of CD11b^{+}Ly6G^{-} cells (p=<0.0001) as well whereas IFN-γ does not impact the percentage of CD11b^{+}Ly6G^{-} cells in wildtype cultures (p=0.9940). TET2$^{+/-}$ HSCs only produce a reduced total number of CD11b^{+}Ly6G^{-} cells in response to TGF-β (p=0.0242).


a)
**
***
****
100
50
0
% CD11b+Ly6G-
(of live, single cells)
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
WT
TET2+/-
Cell type +/- treatment

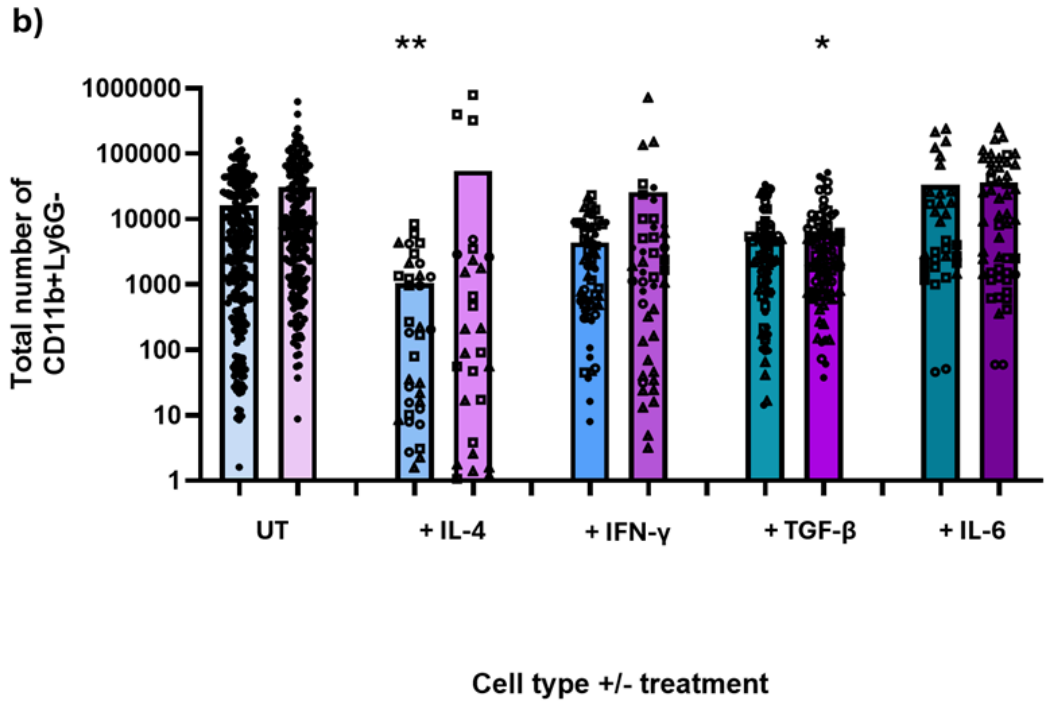

b)
**
*
1000000
100000
10000
1000
100
10
1
Total number of
CD11b+Ly6G-
UT
+ IL-4
+ IFN-γ
+ TGF-β
+ IL-6
Cell type +/- treatment

Figure 17: IFN-γ reduces the proportion of CD11b+Ly6G- cells in TET2$^{+/-}$ cultures

a) Wildtype HSCs produce a reduced percentage of CD11b^{+}Ly6G^{-} cells after treatment with IL-4 (p=0.0059) as do TET2$^{+/-}$ HSCs (p=0.0004). In response to IFN-γ, the percentage of CD11b^{+}Ly6G^{-} produced by wildtype HSCs does not change (p=0.9940) whereas TET2$^{+/-}$ cultures have a reduced percentage of CD11b^{+}Ly6G^{-} (p=<0.0001) **b)** TET2$^{+/-}$ HSCs produce a reduced total number of CD11b^{+}Ly6G^{-} in response to TGF-β (p=0.0242). Wildtype HSCs produce a reduced total number of CD11b^{+}Ly6G^{-} cells when treated with IL-4 (p=0.0021). In response to IL-4, TET2$^{+/-}$ HSCs produce a greater total number of CD11b^{+}Ly6G^{-} compared to wildtype IL-4 treated (p=0.003). Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

3.3.3 TET2$^{+/-}$ cultures express more Sca-1 than wildtype cultures

As Sca-1 was included in the panel, we noticed that Sca-1 expression was higher in TET2$^{+/-}$ untreated cultures than wildtype untreated cultures (p=0.0204) [as shown in the appendix]. The gating strategy is shown in **Figure 18**. We gathered the untreated data from every experiment conducted using 10-day cultures and calculated the significance using an unpaired t-test with Welch's correction, as shown in **Figure 19**. We found that TET2$^{+/-}$ untreated HSCs produce a greater percentage of Sca-1 positive cells than wildtype untreated HSCs (p=0.0003). TET2$^{+/-}$ untreated HSCs also produce a greater total number of Sca-1 positive cells than wildtype untreated HSCs (p=0.0454).

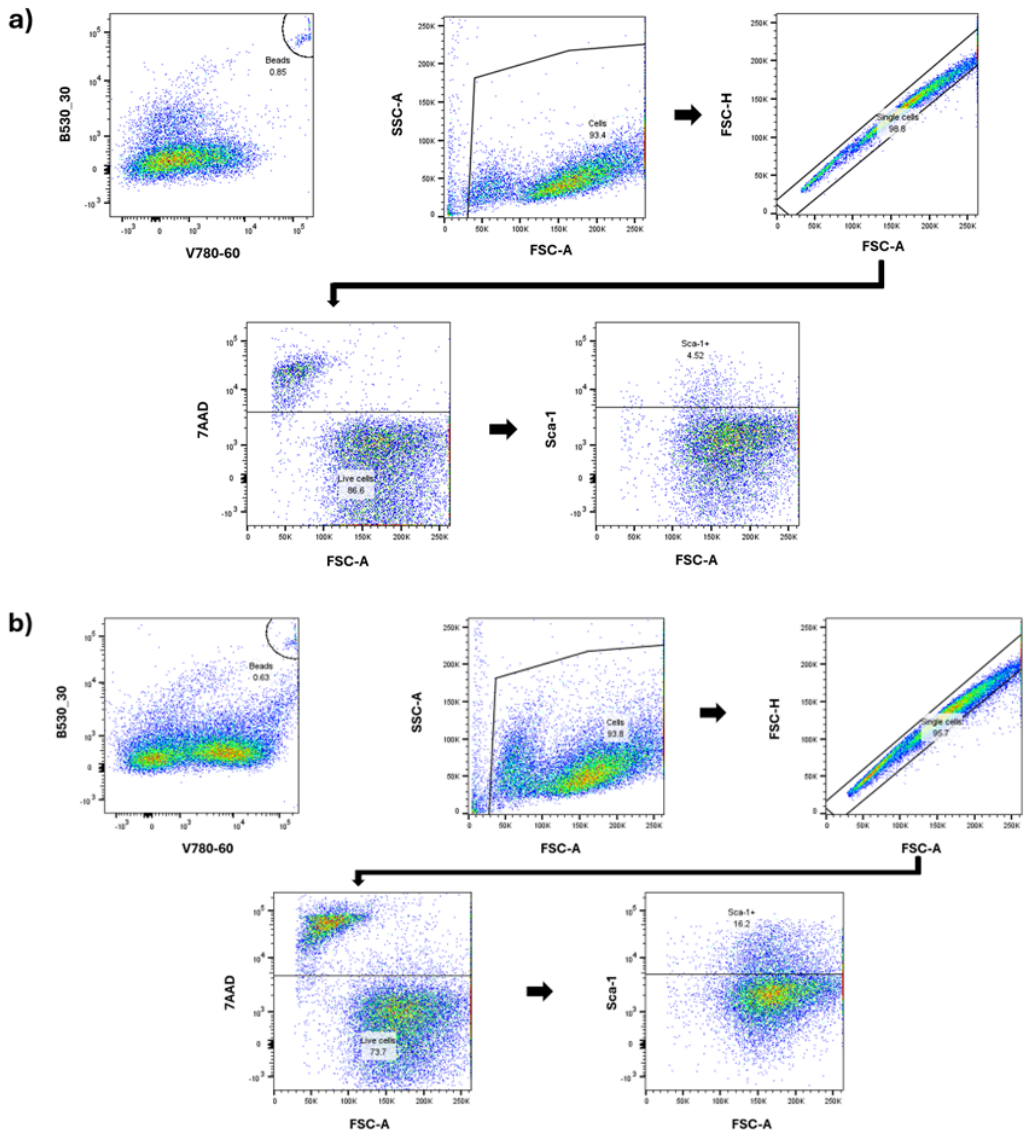


Figure 18: The gating strategy to illustrate TET2$^{+/-}$ cultures have elevated Sca-1 expression

a) The gating strategy used to isolate and quantify Sca-1 positive cells in wildtype untreated cultures. **b)** The gating strategy used to isolate and quantify Sca-1 positive cells in TET2$^{+/-}$ untreated cultures showing elevated expression of this marker.

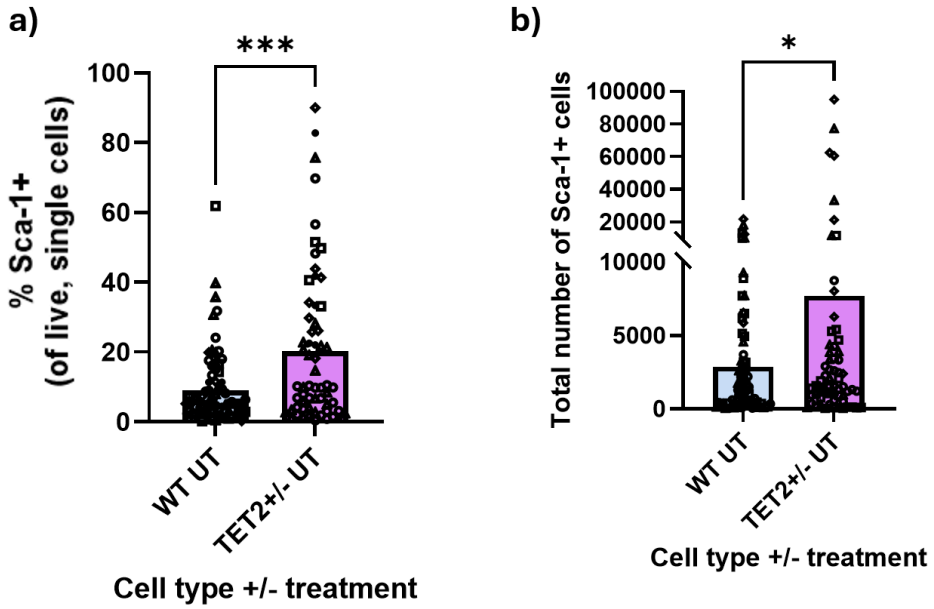


Figure 19: TET2$^{+/-}$ cultures have elevated Sca-1 expression

a) TET2$^{+/-}$ untreated cultures have a greater percentage of Sca-1 positive cells than wildtype untreated cultures (p=0.0003, unpaired t-test with Welch's correction). **b)** TET2$^{+/-}$ untreated HSCs produce a greater number of Sca-1 positive cells after 10-days in culture than wildtype untreated HSCs (p=0.0454, unpaired t-test with Welch's correction). * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=12

3.4 TET2$^{+/-}$ HSC's do not display reduced colony forming efficiency in response to cytokines

3.4.1 IL-4 has a selective negative impact on TET2$^{+/-}$ HSC viability

With the aim of assessing survival in response to the different cytokines compared between wildtype and TET2$^{+/-}$, we isolated HSCs from wildtype and TET2$^{+/-}$ mice using ESLAM FACS and then cultured them for 10 days with or without the addition of cytokines. The cultures were then analysed using flow cytometry and the live/dead stain used was 7AAD. We hypothesised that wildtype would be more sensitive to death induced by the cytokines than TET2$^{+/-}$ so would have a reduced percentage live in the treated cultures whereas TET2$^{+/-}$ would not.

Through these analyses, we found that IL-4 reduced the viability of wildtype cultures (p=<0.0001) but not TET2$^{+/-}$ cultures (p=0.2370) and TET2$^{+/-}$ treated cultures have an

increased viability compared to wildtype treated cultures (p=0.0114), as shown in **Figure 20**. We also demonstrated that, in response to IFN-γ, both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) cultures suffer a reduced live cell percentage. We observed no reduction in percentage live in response to TGF-β for either wildtype (p=0.9572) or TET2$^{+/-}$ (p=0.9995) cultures. There was also no significant difference in percentage live in response to IL-6 for either wildtype (p=0.9556) or TET2$^{+/-}$ (p=0.9976).

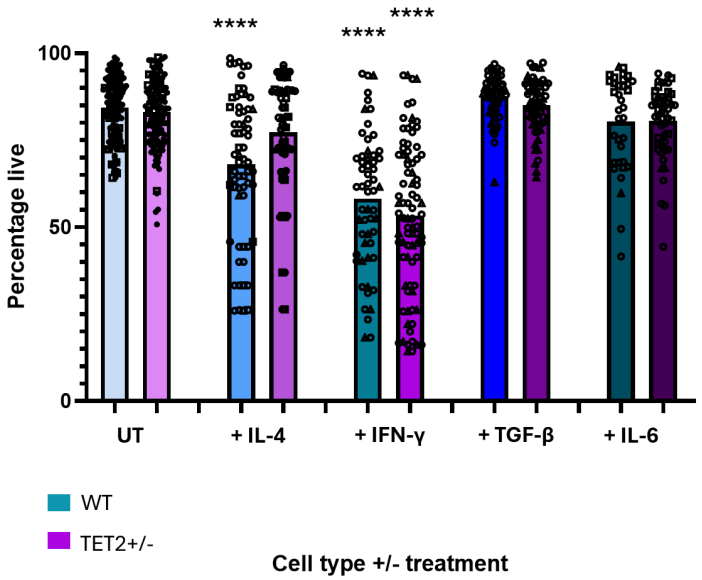


Figure 20: TET2$^{+/-}$ cultures do not exhibit reduced viability in response to IL-4

The percentage live was reduced in response to IL-4 for wildtype cultures (p=<0.0001) but not for TET2$^{+/-}$ cultures (p=0.2370) and TET2$^{+/-}$ treated cultures have an increased viability compared to wildtype treated cultures (p=0.0114). The percentage live was reduced in response to IFN-γ treatment for both wildtype (p=<0.0001) and TET2$^{+/-}$ (p=<0.0001) cultures. Neither wildtype (p=0.9572) nor TET2$^{+/-}$ (p=0.9995) cultures have reduced viability in response to TGF-β treatment. There is also no change in percentage live in response to IL-6 for either wildtype (p=0.9556) or TET2$^{+/-}$ (p=0.9976). Wildtype is shown in blue and TET2$^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12), indicated by differently shaped points [All comparisons displayed on the graph are

compared to untreated of the same genotype, unless otherwise indicated]

3.4.2 TET2$^{+/-}$ HSCs retain colony forming capacity in response to IL-4 and IFN-γ

In order to assess the colony forming efficiency of HSCs in response to cytokines compared between wildtype and TET2$^{+/-}$, the percentage of single cells that formed colonies was calculated for each genotype and condition. We found that wildtype HSCs produced fewer colonies in response to IL-4 than untreated (p=<0.0001) and fewer than TET2$^{+/-}$ IL-4 treated HSCs produced (p=0.0001), as shown in **Figure 21**. TET2$^{+/-}$ IL-4 treated HSCs do not produce fewer colonies than TET2$^{+/-}$ untreated HSCs (p=>0.9999). In response to IFN-γ, wildtype HSCs produce fewer colonies than untreated (p=0.0002) whereas TET2$^{+/-}$ IFN-γ treated HSCs do not produce fewer colonies than TET2$^{+/-}$ untreated (p=0.3597). We found no changes in the percentage of cells that formed colonies in response to TGF-β or IL-6 for wildtype (p=0.7742 and p=9885, respectively) or TET2$^{+/-}$ (p=0.9998 and p=9758, respectively).

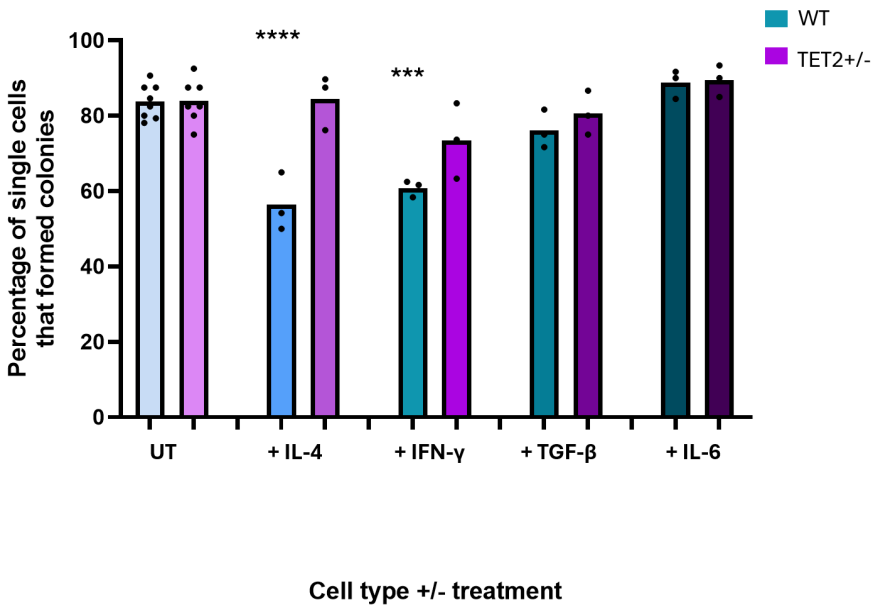


Figure 21: TET2$^{+/-}$ HSCs do not demonstrate reduced colony forming efficiency in response to IL-4 and IFN-γ

The percentage of single cells that formed colonies was calculated by dividing the total number of colonies formed by the total number of cells sorted for that condition for each experiment. We found that wildtype HSCs produced fewer colonies in response to IL-4 than untreated (p=<0.0001) and fewer than $TET2^{+/-}$ IL-4 treated HSCs produced (p=0.0001). $TET2^{+/-}$ IL-4 treated HSCs do not produce fewer colonies than $TET2^{+/-}$ untreated HSCs (p=>0.9999). In response to IFN-γ, wildtype HSCs produce fewer colonies than untreated too (p=0.0002) whereas $TET2^{+/-}$ IFN-γ treated HSCs do not produce fewer colonies than $TET2^{+/-}$ (p=0.3597). Wildtype is shown in blue and $TET2^{+/-}$ in purple. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001 N=3 per treatment (12) [All comparisons displayed on the graph are compared to untreated of the same genotype, unless otherwise indicated]

4 Discussion and Conclusions

In this thesis, we set out to establish whether TET2 mutant HSCs were differentially affected by different levels of cytokines as a potential mechanism by which they acquire clonal advantage. Through the experiments described, we were able to demonstrate that TET2 mutant HSCs were hyporesponsive to IL-4, do not divide faster and do not suffer reduced viability in response to the cytokines tested.

Our findings confirm that TET2 mutant HSCs are less responsive to inflammatory cytokines than wildtype HSCs [(234,253,254)]. We showed that TET2 mutant HSCs do not respond to as low a concentration of IL-4 as wildtype HSCs and are not prompted to differentiate or die in response to IL-4 even at the highest concentration. We showed that when wildtype HSCs are treated with a low concentration of IL-4, their colony forming efficiency decreases whereas the colony forming efficiency of $TET2^{+/-}$ HSCs does not decrease at all, even at a high concentration. Wildtype HSCs lack the protective epigenetic mechanisms that allow $TET2^{+/-}$ HSCs to form colonies through clonal expansion even in the presence of inflammatory cytokines [(255,256)]. Wildtype cultures have an altered percentage of and total number of ELSKs even at low concentrations of IL-4 whereas $TET2^{+/-}$ cultures show no changes in the percentage of or total number of ELSKs even at high concentrations. However, the use of ELSK gating strategy to identify HSCs has not yet been validated for use with the 10-day culture system while it has been validated for longer cultures [(73,257,258)]. As a result, it was important to confirm whether this effect was present with the LSK gating strategy as well, even though this LSK population is known to include progenitors [(259)]. LSK populations showed the same trend in response to IL-4 of a reduction for wildtype cultures but no change for $TET2^{+/-}$ cultures. IL-4 is known to promote differentiation of wildtype HSCs but inhibit particular differentiation pathways and promote apoptosis [(260–263)]. In wildtype

cultures, at low concentrations, the percentage of ELSKs increases and then decreases back to the level of untreated at a high concentration of IL-4. This could be due to low doses of IL-4 affecting progenitor and precursor cells before HSCs get affected by higher concentrations. Additional research involving IL-4 has shown that progenitor cells may be sensitive to the cytotoxic effects of IL-4 at lower concentrations [(264)]. $TET2^{+/-}$ cultures appear to not demonstrate any of these effects as there are no changes in the percentage of or total number of ELSKs or LSKs at any concentration of IL-4. This could be because $TET2^{+/-}$ HSCs are less likely to differentiate into progenitors in culture anyway [(265)]. Despite this, total cell number in response to IL-4 is reduced for both wildtype and $TET2^{+/-}$ colonies as the ability of single HSCs to form large colonies is reduced for wildtype HSCs from the lowest concentration tested whereas this reduction only happens for $TET2^{+/-}$ HSCs at higher concentrations.

Through our analyses on the use of IL-6 at a range of concentrations, we determined that wildtype HSCs respond best at high concentrations. In the healthy state, IL-6 has many beneficial effects on haematopoiesis and regeneration [(266)]. IL-6 is known to be produced by myeloma and sarcoma cell lines and the upregulation of its signalling cascades plays an important role in the development and progression of cancer [(266,267)]. These cell lines have been shown to be dependent on IL-6 [(268,269)]. And whilst primary HSCs are not dependent on IL-6 we did find them responsive to the highest concentration of IL-6 commonly used in culture. IL-6 acts through an autocrine IL-6-mediated growth loop which promotes transition into S-phase of the cell cycle [(267)]. The IL-6/GP130/STAT3 pathway through which IL-6 promotes proliferation has been extensively studied due to its role in tumour formation [(270)]. The concentrations commonly known to be secreted by human cells are up to 21.8 ng/ml and this has been shown to negatively influence survival from conditions such as pulmonary arterial hypertension compared with healthy control levels of up to 14.8 ng/ml [(271)].

Proliferation of many cell types is known to be inhibited by IL-4 through blockage of the cell cycle preventing cells entering S and G2/M phase [(272,273)]. We were able to corroborate this inhibition of proliferation through our experiments but IL-4 is also able to promote proliferation when in conjunction with other growth factors such as GM-CSF [(274)]. Previous studies suggested that TET2 mutant HSCs could be less sensitive to the inhibition of proliferation caused by some cytokines, but we did find reduced total cell number and reduced colony area in response to IL-4 for $TET2^{+/-}$ as we did wildtype [(275)]. Studies investigating the effect of IFN-γ on wildtype HSCs showed impaired proliferation [(276)]. Other studies using different experimental techniques have again shown that TET2 mutant HSCs should be less sensitive to the negative effects of inflammatory cytokines [(234,275,277,278)]. However, we found that $TET2^{+/-}$ HSCs do not divide, increase total cell number and colony area as well in response to IFN-γ just like wildtype. TGF-β is known to regulate many aspects of HSC behaviour including self-renewal so we wanted to assess the total number of

cells within cultures in response to this cytokine to determine whether $TET2^{+/-}$ would show any alterations in proliferation more so than wildtype [(279)]. However, the response of HSCs to TGF-β is biphasic, with low concentrations promoting proliferation but high concentrations inhibiting proliferation [(280)]. We had selected a concentration of 1 ng/ml which is a low concentration and the media contained IL-11 which promotes proliferation and this could explain why we found no additional proliferation in the treated wells compared with the untreated wells. Despite no change in cell number, we did observe a slight decrease in colony area in response to TGF-β which may be because cells packed closer together in response to treatment for both wildtype and $TET2^{+/-}$ cultures. IL-6 increases the total cell number of both wildtype and $TET2^{+/-}$ cultures in the same way that IL-11 (a component of ten-day expansion culture media) does. This is because both of these cytokines activate the same receptor, specifically β-receptor glycoprotein 130, and the same signalling cascades [(281)]. These pathways are predominantly the mitogen-activated protein kinase (MAPK)-cascade and the Janus kinase/signal transducer in addition to the activator of transcription (Jak/STAT) pathway [(281)]. It would be interesting to determine whether IL-6 promotes HSC proliferation to the same extent without IL-11 in the media. Despite the increase in total cell number, in response to IL-6, culture area for both genotypes was slightly reduced. This could be again because the cells stack into layers more closely together than untreated. This reduction in colony area was less substantial for $TET2^{+/-}$ cultures than wildtype cultures though so $TET2^{+/-}$ HSCs may be less susceptible to this process.

By counting cells to assess the divisional kinetics of wildtype and $TET2^{+/-}$ HSCs untreated and in response to each of the cytokines, we confirmed that wildtype cells divide significantly faster in response to the cytokines compared with their untreated counterparts. This is because all inflammatory signals promote the rapid activation and differentiation of HSCs into more committed progenitors which can then divide much more rapidly [(282)]. Cytokines such as IL-4 are known to affect progenitors to a greater extent than HSCs but can promote division through rapid differentiation [(283)]. We have demonstrated that this effect can begin just after FACS ESLAM isolation so potentially HSCs are stimulated to divide just like progenitors. It would be advisable with IL-4 to confirm that the progenitors are not releasing something that HSCs do respond to through analysis of the media at regular intervals using techniques such as ELISA and proteomics. IFN-γ is known to activate quiescent HSCs and promote their rapid division and differentiation into downstream effector immune cells as part of emergency haematopoiesis [(119)]. TGF-β is known to promote rapid HSC division through the canonical Smad signalling as these transcription factors can promote cell division [(279,284)]. IL-6 is known to have a similar effect to IFN-γ by promoting HSC activation and stimulating emergency haematopoiesis as well [(285)]. This rapid response has been known to promote the fascinating process of immunological memory within HSCs [(286)]. The exact mechanisms of this process are not yet fully understood within HSCs but it is thought to involve alterations of chromatin organization within topologically associated

domains (TADs), reprogramming of cellular metabolism and transcription of long non-coding RNAs (lncRNAs) [(286)]. This epigenetic reprogramming is conserved and can act like scars across DNA that can alter responses to the same stimuli in future [(286)]. Repeated exposures to inflammation can promote the concept of inflamm-aging through the mechanisms described above [(287)]. It would be fascinating to determine the impact of TET2 mutations on these mechanisms as my data could imply that these mechanisms may not be activated in $TET2^{+/-}$ HSCs. We found that $TET2^{+/-}$ HSCs do not divide faster in response to any of the cytokines studied throughout this project. This could be due to their reduced sensitivity to the cytokines and delayed differentiation as a response, as is widely known, but it would be beneficial to understand the particular mechanisms involved with their divisional kinetics. Malignant cells, such as those possessing a TET2 mutation, are known for hypomethylation associated with genomic instability in addition to promoter hypermethylation which contributes to the inactivation of tumour suppressors along with genes responsible for control of the cell cycle and repair [(174–176)]. It is also known that many signalling cascades are altered in response to TET2 mutations, but it would be important to understand the particular alterations that may impact their rate of division and their progression through the cell cycle in response to cytokines using techniques such as proteomics at a range of time points post cytokine treatment.

Through the analysis of mice with targeted disruption of the TET2 catalytic domain, TET2 has been found to be a critical regulator of self-renewal and differentiation of HSCs [(196)]. These data confirm this finding as, in all experiments, TET2 retained a higher total number of ELSKs. Defining a HSC immunophenotypically however is problematic and it would be advisable to confirm their presence using transplantation (288). It would also be interesting to perform these with the treated HSCs in order to determine whether the function of the HSCs is altered as well as their quantity. Further experiments that would be useful to deepen understanding of the functional consequences of these cytokines on HSCs would be to perform CFU assays using the HSCs from the endpoint of the 10 day cultures treated wells. Defining any cell type using immunophenotyping after treatments is not optimal so it would also be advisable to confirm the expression of markers using RNA sequencing for known targets or proteomics to remove the bias associated with RNA sequencing [(289)].

TET2 loss is also known to bias the differentiation of HSCs towards the myeloid lineage [(246)]. This data confirmed this as $TET2^{+/-}$ HSCs produced a greater percentage and total number of myeloid lineage cells (granulocytes and $CD11b^{+}Ly6G^{-}$). These $CD11b^{+}Ly6G^{-}$ cells could be $CD11b^{+}$ (myeloid) progenitors, monocytes or monocyte-derived macrophages [(290–293)]. They are likely monocytes or monocyte-derived macrophages but additional markers would need to be included in the panel to provide more certainty of cell type, especially in the context of cytokine treatments and TET2 mutations [(293,294)]. Research has highlighted the reasons for this myeloid bias as the altered methylation patterns

leading to hypermethylation through co-mutations [(220)]. This is why the TET2 mutations are commonly observed in stem cells from patients with myeloid malignancies preceding the JAK2 V617F mutation often observed in more differentiated progeny from individuals with AML [(213)]. As a result of additional mutations in more differentiated progeny, it could be fascinating to investigate the epigenetic and genetic states of cells from $TET2^{+/-}$ cultures at the endpoint and determine whether any of the cytokine treatments lead to differences in the additional mutations that arise. For example, based on my finding that TGF-β reduces the proportion of myeloid cells produced by $TET2^{+/-}$ HSCs, it would be interesting to see whether the TGF-β treated $TET2^{+/-}$ HSCs are genetically and epigenetically identical to untreated $TET2^{+/-}$ HSCs. This could be achieved by performing DNA methylation profiling on these two cell types [(295)].

TET2 deficient mice are known to display a more severe inflammatory phenotype which could explain why, even in untreated cultures, $TET2^{+/-}$ ESLAM sorted cells after 10 days have a greater number of Sca-1 positive cells. This inflammatory phenotype is due to upregulation of mediators of inflammation such as IL-6 as TET2 is no longer present to repress transcription of the genes involved in their expression [(211)]. Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL-6. [(211)]. In the mice used in these experiments, the inflammatory phenotype is usually only noticeable later in their lives and the mice used in these experiments were still young. It would be interesting to determine if this inflammatory phenotype is as a result of the underlying genetic differences compared with wildtype or if this is due to increased inflammation exposure throughout development. This could be achieved by inducing the same TET2 mutation in HSCs from older wildtype mice and comparing their Sca-1 expression and cytokine responses. Previous research attempting this in tumour tissue myeloid cells did discover a non-cell-intrinsic function of Tet2 which led to tumour promotion (294). This highlights the importance of understanding the role of Tet2 in each cell type so it would be worth attempting the same in a range of different cell types.

Previous research has primarily focused on the impact of cytokines on immune cells [(296,297)] but the data here suggest that HSCs are able to respond to the cytokines themselves, adding to other recent work using different experimental techniques [(133,179,298)]. This could imply that cellular responses to infections and aging occur much earlier in haematopoiesis than was previously expected. It has been shown that IFN-γ injections into live mice are able to induce Fas-mediated apoptosis in both HSCs and HSPCs [(299)]. The divisional kinetics data that I have collected show that potentially a different mechanism is involved ex-vivo as the effects of this Fas-mediated apoptosis should be visible within twelve hours at the latest, although cytokines in the media may have contributed to this delay through promoting cell division [(300,301)]. As $TET2^{+/-}$ cells appeared to be first to suffer cell death in response to IFN-γ, it would be beneficial to elucidate the mechanisms behind this altered timing.

Previous research in-vivo did observe a reduction in the numbers of leukaemia initiating cells (stem-like cells) in response to IL-4 [(302)] and this has been found to be due to alterations in signalling cascades, leading to excessive apoptosis in HSCs in response to stress [(133)]. However, the divisional kinetics data appears to suggest that this response cannot be immediate and likely affects the more differentiated progenitor cells as the HSCs were able to divide normally for at least three days. The mechanisms behind this would be worth understanding in more detail. In order to understand the potential differences in cytokine signalling of HSCs compared with progenitors, associated proteins involved in the signalling cascades could be identified by immunoprecipitation followed by mass spectrometry [(303)]. This could identify stark differences in the components of the signalling cascades but it could be more beneficial to identify subtle differences through techniques such as optogenetics or proteomics [(304,305)].

A further consideration for this project [*all summarised in the appendix*] is the assessment of viability using flow cytometry is not optimal due to hypocellular specimens (such as those in the treated wells) leading to inaccurate viability [(306)]. It would therefore be advisable to follow up these experiments with techniques such as ATP assays [(307)]. This would be necessary to validate the findings relating to $TET2^{+/-}$ cultures not experiencing death in response to IL-4 compared with a substantial decrease in percentage live for wildtype and the decreases in percentage live in response to IFN-γ for both wildtype and $TET2^{+/-}$.

The ability of single HSCs to form colonies is not altered in response to TGF-β or IL-6 for both wildtype and $TET2^{+/-}$. TGF-β has been known to inhibit the ability of single HSCs to form colonies when combined with other factors such as thrombopoietin (TPO) [(308)]. This effect is because TGF-β is known to be a negative regulator of HSCs and HSPCs in vitro [(309)]. However, we were not able to demonstrate the same effect with TGF-β impairing colony formation. This may be due to the dose-dependent effects described previously as we used a lower concentration of this cytokine [(280)]. Specifically, the low concentration associated with activation of STAT proteins as opposed to phosphorylation of SMAD 3, could potentially not inhibit colony formation as some STAT proteins can promote cell proliferation once activated [(280,310)]. IL-6 has been shown to increase colony forming efficiency for macrophages in combination with M-CSF [(311)]. The IL-6/GP130/STAT3 pathway through which IL-6 promotes colony formation has been extensively studied due to its role in tumour formation [(270)]. We were able to demonstrate the same for HSCs with increased colony size and a lack of reduction in colony forming efficiency compared to untreated. IL-4 and IFN-γ result in a sizeable reduction in colony forming efficiency for single wildtype HSCs but does not impair this ability for single $TET2^{+/-}$ HSCs. This demonstrates that IL-4 and IFN-γ can impact the survival of many types of cells (after the first three days, as described previously) and impair colony formation for wildtype cells [(312–314)]. The lack of an impact on $TET2^{+/-}$ may be due to the improved tolerance of cytokines and ability to persist in

inflammatory environments [(196,250,275,315,316)]. $TET2^{+/-}$ HSCs are able to persist in inflammatory environments due to reduced susceptibility to cytokine induced down regulation of the genes involved in self-renewal [(275)]. Interestingly with IFN-γ however, $TET2^{+/-}$ appears to be the first to experience death from the divisional kinetics data but despite this experiences no colony forming efficiency reduction by day 10. This shows that TET2 mutation mediated resistance to inflammatory cytokines may not be cell-intrinsic and could be the result of compensatory mechanisms developed through exposure to elevated concentrations of cytokines. It would be beneficial to study the expression of particular genes at different time points in response to cytokine treatment. This could be achieved through RNA sequencing of the cells and it would be advisable to analyse the substances being released by the cells by analysing the supernatant from culture media using ELISAs [(317,318)].

In conclusion, through this project we have confirmed previous work demonstrating the reduced responsiveness of TET2 mutant HSCs to many inflammatory cytokines. We showed that aspects of this reduced responsiveness of $TET2^{+/-}$ HSCs begins almost immediately as they are not prompted to divide faster in response to the cytokine treatments whereas wildtype HSCs do for all of the cytokines studied. However IL-4 and IFN-γ do appear to reduce proliferation for both wildtype and $TET2^{+/-}$ cultures overall. $TET2^{+/-}$ cultures retain ELSKs, LSKs and ESAM and EPCR double-positive stem cells in culture despite the presence of the differentiation promoting IL-4. However, in response to TGF-β and IL-6, both wildtype and $TET2^{+/-}$ HSCs differentiate. IFN-γ appears to not prompt differentiation of either wildtype or $TET2^{+/-}$ HSCs. We also showed that TGF-β reduces the proportion of $TET2^{+/-}$ cultures that are myeloid lineage cells to a level comparable to wildtype untreated. We also found that $TET2^{+/-}$ appears to have a more inflammatory phenotype with increased Sca-1 expression in untreated wells. We demonstrated that wildtype cultures have a reduced percentage live in response to IL-4 and IFN-γ but this reduced viability was only observed in $TET2^{+/-}$ cultures in response to IFN-γ. Finally, we demonstrated that wildtype HSCs show a reduced colony forming efficiency in response to IL-4 and IFN-γ but $TET2^{+/-}$ HSCs do not demonstrate a reduced colony forming efficiency in response to any of the cytokines we tested.

As detailed previously, the haematopoietic factors that HSCs are exposed to can establish epigenetic programs that are able to contribute to the cell fate decisions made by the HSC for a long time [(191)]. It is therefore important to understand what the phenotypic changes are from each insult to be able to understand why HSCs may become more biased in response to threats. This is the concept of immunological memory and this in HSCs allows for longer lasting improvements in immune responses as most immune cells typically have a short lifespan [(319)]. This is fundamental to orchestrate immune plasticity through altering the functional

capabilities of progeny that haven't previously encountered the inflammatory environments or infections [(319)]. The impacts of TET2 mutations on these epigenetic and metabolic changes in response to infection and inflammation have not been studied. It is possible that these changes are prevented as a result of reduced responsiveness to cytokines of TET2 mutant HSCs. This would be important to investigate as therapeutic targets for the conditions associated with these mutations could be identified through this novel approach. It would be therefore advisable to follow this work by investigating the epigenetic and metabolic processes that are currently being investigated for understanding immunological memory of HSCs in the context of TET2 mutations to better understand the prognosis for myeloid malignancies in young adults and children, even though there is lower incidence of these mutations in childhood leukaemias [(320)].

Future work summarised

I would of course recommend repeating each experiment to achieve at least N=5 but based on the initial data provided, I would recommend testing the impact of IL-6 on HSCs in 10-day expansion media lacking IL-11. I would also recommend using techniques such as ELISA and proteomics on the culture media at regular intervals to determine whether progenitors are releasing additional substances that the HSCs are able to respond to or confirm that the HSCs are responding to the cytokines themselves. It would also be fascinating to determine the impact of TET2 mutations on the mechanisms of inflamm-aging and perform proteomics on TET2 mutant HSCs at regular intervals post cytokine treatment to study the impact of TET2 mutations on the divisional kinetics of cytokine treated HSCs. Further experiments that would be useful to deepen understanding of the functional consequences of these cytokines on HSCs would be to perform CFU assays using the HSCs from the endpoint of the 10 day cultures treated wells. As defining a HSC immunophenotypically is problematic, it would also be advisable to confirm marker expression using RNA sequencing or proteomics and confirm their "stemness" using transplantations with the option to include cytokine treatment as a variable to determine the impact of these substances on stem cell function. It could be fascinating to investigate the epigenetic and genetic states of cells from $TET2^{+/-}$ cultures at the endpoint of 10-day cultures using techniques such as DNA methylation profiling and determine whether any of the cytokine treatments lead to differences in the additional mutations that arise. An arguably essential project would be to induce the same TET2 mutation in HSCs and other cell types from older wildtype mice and compare their Sca-1 expression and cytokine responses as this would allow us to determine whether the altered responses are due to the intrinsic genetic differences or due to increased exposure to inflammation throughout development. As $TET2^{+/-}$ cells appeared to be first to suffer cell death in response to IFN-γ, it would also be beneficial to elucidate the mechanisms behind this altered timing. In order to understand the potential differences in cytokine signalling of HSCs compared with progenitors, associated proteins involved in the signalling cascades could be identified by

immunoprecipitation followed by mass spectrometry. This could identify stark differences in the components of the signalling cascades but it could be more beneficial to identify subtle differences through techniques such as optogenetics or proteomics. As assessing viability with flow cytometry is not ideal, it would be advisable to follow up these experiments with techniques such as ATP assays to confirm the impact of each cytokine on cell death. Furthermore, it would be beneficial to study the expression of particular genes at different time points in response to cytokine treatment. This could be achieved through RNA sequencing of the cells of both genotypes to elucidate altered response mechanisms.

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7. Appendix

Interferon gamma is known to induce Sca-1 expression on a range of different

progenitor cells [(126,321)]. Therefore, to confirm the IFN-γ was causing the expected induction of Sca-1 expression after 10 days in culture. We compared its expression between the treated and untreated wells for both wildtype and $TET2^{+/-}$ cultures and the results are shown in **figure 22**. We confirmed that Sca-1 expression was significantly increased in response to IFN-γ for both wildtype (p=<0.0001) and $TET2^{+/-}$ (p=<0.0001) cultures. This helps to demonstrate that the IFN-γ was functional and the cells were responsive. We also found, through this analysis, that $TET2^{+/-}$ cultures have an increased percentage of Sca-1 positive cells compared with wildtype untreated (p=0.0204). Significance was calculated using a Brown-Forsythe and Welch ANOVA test.

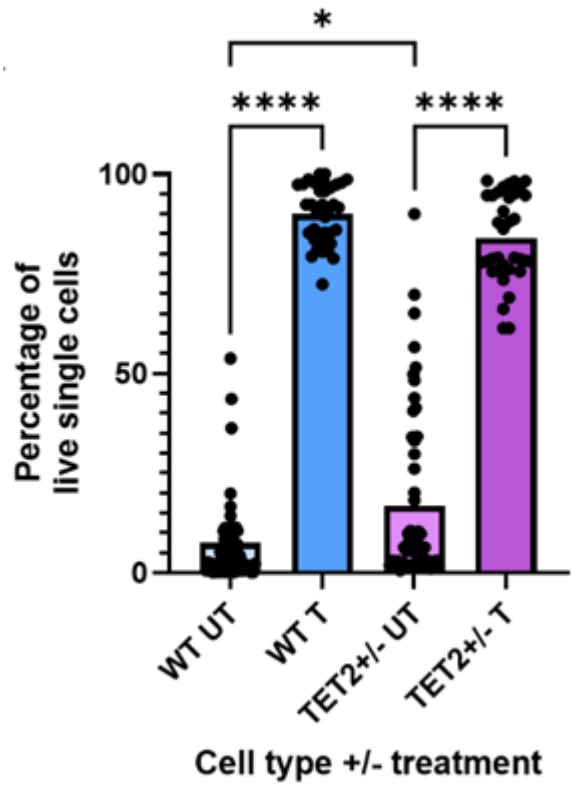

Figure 22: IFN-γ increases Sca-1 expression substantially
Sca-1 expression was measured as a percentage of live single cells after 10 days in culture of ESLAM sorted cells. The expression of this marker was increased in response to IFN-γ for both wildtype (p=<0.0001) and $TET2^{+/-}$ (p=<0.0001) cultures. $TET2^{+/-}$ cultures also have an increased percentage of Sca-1 positive cells compared with wildtype untreated (p=0.0204). Significance was calculated using a Brown-Forsythe and Welch ANOVA test. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001

Previously, no difference was found in the divisional kinetics between wildtype and $TET2^{+/-}$ HSCs [(35)]. As a result, we wanted to confirm that this was the case for the

cells in our culture too. We plotted this in **figure 23** and performed a Fisher's exact test to determine significance**.** We were able to confirm that there was no significant difference in the rate of division between wildtype and TET2$^{+/-}$ untreated HSCs (p=0.4154).

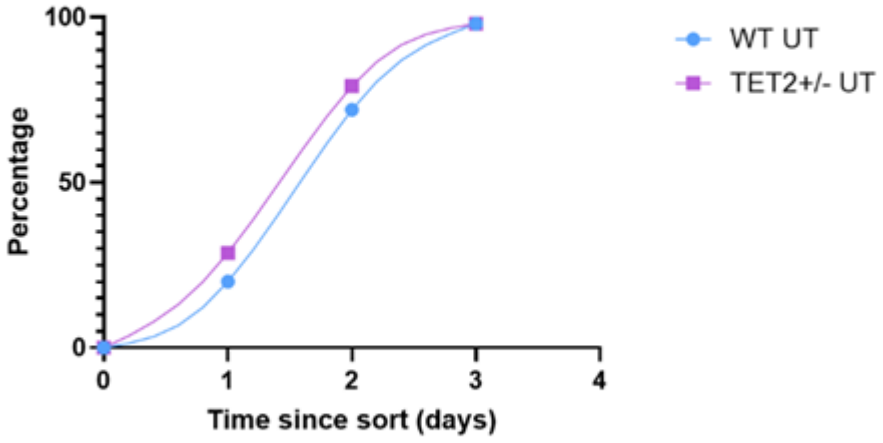

Figure 23: There is no significant difference in the rate of division between wildtype and TET2$^{+/-}$ untreated HSCs
The percentage of cells that had undergone the first division on days 1-3 since the sort was calculated for each genotype in untreated wells. Significance was calculated using Fisher's exact test. No difference was found between the percentage of wildtype and TET2$^{+/-}$ HSCs that had undergone division over the first 3 days (p=0.4154).

Mouse details
The iliac and femur were collected from the following mice:

Table 2: Details of the mice used for the experiments in this thesis

Genotype	Strain	Age (weeks)	Sex	Experiment
WT	Fgd5	10	Female	IL-6 dose test
WT	Fgd5	8	Male	IL-4 dose test
TET2+/-	Tet2	8	Male	IL-4 dose test
WT	B6.CD45.1	9	Male	IL-4 one
TET2+/-	Tet2	8	Male	IL-4 one
WT	B6.CD45.1	11	Male	IL-4 two
TET2+/-	Tet2	9.9	Male	IL-4 two
WT	C57Bl/6J	9	Male	IL-4 three
TET2+/-	Tet2	9.9	Female	IL-4 three
WT	B6.CD45.1	9	Male	IFN-γ one
TET2+/-	Tet2	8	Male	IFN-γ one
WT	B6.CD45.1	11	Male	IFN-γ two
TET2+/-	Tet2	10	Male	IFN-γ two
WT	C57Bl/6J	9	Male	IFN-γ three
TET2+/-	Tet2	9.9	Female	IFN-γ three
WT	C57Bl/6J	8.6	Female	TGF-β one
TET2+/-	Tet2	10.9	Female	TGF-β one
WT	Fgd5	10.4	Female	TGF-β two
TET2+/-	Tet2	10.9	Female	TGF-β two
WT	Fgd5	10.4	Female	TGF-β three
TET2+/-	Tet2	10.9	Female	TGF-β three
WT	Fgd5	8.7	Female	IL-6 one
TET2+/-	Tet2	8.6	Female	IL-6 one
WT	Fgd5	8.7	Male	IL-6 two
TET2+/-	Tet2	8.7	Male	IL-6 two

WT	C57Bl/6J	9	Male	IL-6 three
TET2+/-	Tet2	12	Female	IL-6 three

The different repeats are illustrated as different shaped points on each of the figures throughout this thesis.

The limitations of this work summarised

Area	**Limitation**
Replicates	In some cases there is quite high variability of replicates which could be due to technical error (such as sorting too few cells) but also in some cases there could be a biological reason for this variability such as different cellular states etc (explained further below).
Divisional kinetics	When counting cells, it is difficult to identify, quantify and distinguish between living or dead non-divided cells (this also relates to the assessment of viability). The methods used in this project struggle to differentiate a scenario where cell division and cell death are in balance. Specific markers are needed to simultaneously assess division history. There can also be a lag between cell division and the onset of death, or vice versa, depending on the stimulus. This makes precise temporal correlation between division events and death events challenging to capture in a single measurement point.
Bias in culture	In culture, robust, rapidly dividing cells may be selected for, meaning measurements do not necessarily reflect the behavior of the full, diverse cell population found in living tissue, which includes cells with varying replicative potentials.
Assessment of viability	Cells can die by different mechanisms (such as apoptosis or necrosis) and each has different physical properties and molecular markers. Measuring a

	mixed population of cells that died via various pathways complicates the analysis as in this project I only used 7AAD positivity to distinguish dead cells. There is also the issue with hypocellular specimens (such as those in the treated wells) leading to inaccurate viability measurements. This highlights how the precise definition of cell death can be complex. A cell might be committed to death (apoptotic) but still technically be "alive" for some time, making endpoint assays less precise about the exact timing relative to the last division event.
Forcibly stimulating cell cycling in culture	Forcibly stimulating quiescent cells into the cell cycle in a culture presents several significant challenges to experimental interpretation, primarily due to cellular heterogeneity and the fact that the in vitro environment does not fully replicate the complex in vivo context. Even within a genetically identical population of cells stimulated under the same conditions, individual cells exit quiescence and re-enter the cell cycle at significantly different paces. This variability makes it difficult to synchronise the cell population and interpret assays, which average the responses of fast-cycling, slow-cycling, and non-responding cells. Quiescent cells exist in a spectrum of "depths," which are influenced by the duration of the arrested state and previous cell cycle history. Deeper quiescent cells require stronger or more prolonged stimulation to re-enter the cycle. Forcing activation therefore might reveal characteristics of a shallow, easily-triggered state rather than the intrinsic properties of a deeply quiescent cell in its natural niche. Relating this to the heterogeneity described above can complicate analysis even further. Forcing cells to cycle may also bypass or impair intrinsic checkpoint

	mechanisms, such as those related to DNA damage repair, which are actively maintained in quiescent cells. This can lead to the study of cells with damaged DNA or abnormal cell cycle progression, rather than the normal physiological process. A further consideration is that cells retain a "memory" of their prior growth and division history, which influences their exit from quiescence. This historical variability can introduce deterministic differences in the cell population's response to stimulation, making it difficult to isolate the effects of the cytokine treatment.
Confirming HSC responses	In vivo, quiescence is regulated by complex intrinsic and extrinsic signals from the cellular niche. In vitro cultures lack this intricate microenvironment, meaning experiments may fail to capture the physiological regulatory mechanisms that normally govern the quiescence-proliferation balance. Through the experiments used here, there is the chance that HSCs have differentiated into cells which respond to the cytokines and subsequently influence HSC behaviour rather than the cytokines themselves directly influencing the HSCs.