Aus dem Walter Brendel Zentrum für Experimentelle Medizin
Biomedizinisches Zentrum der Ludwig-Maximilians-Universität München
Kommissarischer Direktor: Prof. Dr. med. Markus Sperandio

MST1 controls neutrophil homeostasis through regulation of G-CSF receptor signalling

Dissertation zum Erwerb des

Doctor of Philosophy (PhD) in Medical Research

an der Medizinische Fakultät der Ludwig-Maximilians-Universität München

Vorgelegt von

Sergi Masgrau-Alsina

2022

Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Supervisor:	Prof. Dr. med. Markus Sperandio
Second evaluator:	Prof. Dr. Christoph Scheiermann
Third evaluator:	Prof. Dr. Vigo Heissmeyer
Fourth evaluator:	apl. Prof. Dr. Rika Draenert
Dean:	Prof. Dr. Thomas Gudermann
Date of oral defense:	4 0ctober 2022

Acknowledgements

For me, pursuing a PhD has been a journey in which I have met lots of interesting people who have influenced me in the academic but also in my personal life. In the following lines I intend to express my honest gratitude to those without whom this thesis work would not have been possible.

First of all, I would like to thank my supervisor Prof. Dr. Markus Sperandio for giving me the opportunity to develop my doctoral thesis in his laboratory and therefore providing me with excellent tools and equipment for fulfilling it. I appreciate his advice and his guidance during these four years and a half, and above all I thank him for the encouragement and motivation that has given me to conduct the thesis.

I would like to follow thanking my thesis advisory committee members Prof. Dr. Christoph Scheiermann and Prof. Dr. Christian Schulz for the prolific discussions and helping me defining the path of my research.

In my opinion, the working environment is fundamental for a successful development of a project. I couldn't have carried on without the shared knowledge and the friendly surrounding that the senior researchers and doctoral candidates in my working group brought. I am talking about Ina, Roland, Monika, and Heike, and also about Myriam, with whom we shared plenty of good times together. I would like to give a special mention to my colleague Matteo, who quickly after his arrival became my biggest support in the laboratory and also a very good friend. He has been always there either I needed a helping hand to isolate some cells or to cover my back on the busier days. Without his dad jokes my PhD wouldn't have been the same! I am as well grateful to have shared the last period of my doctoral studies with Lou, who I deeply appreciate both scientifically and personally. I really benefited from her tireless effort of sharing her expertise, not to mention her sense of caring for everyone in her surrounding. In her I found a friend to share the *gemütlicher* time with as well as the addiction to *Kleinanzeigen.*

I don't want to miss the opportunity to acknowledge the excellent technical assistance that I've received along these years, first by Susanne Bierschenk and then by Dorothee Gössel, Sabine D'Avis, and Anke Lübeck, and for the great

working atmosphere that Tim, Sebastian, Lilly, Annika, Wang, Katrin, Tobi, Agnes, and all the other students in the laboratory have contributed to.

During my PhD, one of the things that I have enjoyed the most was the excellent platform of seminars and gatherings that the IRTG914 provided, and I sadly missed the most when the pandemic started. Verena was always the person behind the organisation of these events and without her determination in bringing us together and promoting collaboration between the students it wouldn't have been the same. To my fellow PhD candidates, I appreciate the amazing network we created and the good partnership that we established. It was a pleasure to engage in this adventure at the same time as Jasmin, Stephan, Chien-Sin, Sophia, Nikos, Laura, and all the others, and also Anna, with whom I had the pleasure to share the responsibility of being student representatives together.

The perk of fulfilling my doctoral studies in the BMC was the outstanding equipment and technical support that the core facilities of flow cytometry, animal models and bioimaging provide. Of those I would like to mention Dr. Lisa Richter, who enthusiastically introduced me to the world of flow cytometry and always offered her expertise to make things work.

Furthermore, I want to mention my mates from the BMC running club Allwyn and Tamas. I believe that the motivation they transmitted me during the trainings contributed to my performance in the lab – *mens sana in corpore sano*.

Life in Munich crossed several people on my way that ended up being crucial for me on a personal level. For instance, Marta started being a clumsy flatmate that destroyed my shoe rack and now I can't imagine making an important step without first asking her for advice. Concerning Nico, it has been extraordinary to find someone to enjoy coffee breaks between experiments together and feel completely understood. I don't forget about Mark, Ana, Raquel, and all the other friends I've met along the way and made my time in Munich so enjoyable, and neither about Anna P, Anna S, Àlex, Carol, and the rest of friends from my bachelor studies that despite the distance have always supported me.

I per concloure, m'agradaria donar les gràcies a la meva família; al pare, la Núria, l'àvia Pepita, la tieta Contxita i la resta de la família per el recolzament incondicional que sempre he rebut per part seva, però en especial de la meva mare, qui ni tan sols a 1000km de distància he deixat de notar sempre al meu costat.

Abstract

Neutrophils only spend 10-12 hours in the blood vasculature until they get cleared from the circulation. Therefore, continuous release of neutrophils from the bone marrow is indispensable for proper immune function of the organism. Key factor in regulating neutrophil homeostasis is the granulocyte colony-stimulating factor (G-CSF), which not only promotes neutrophil maturation but also favours their release into the circulation. In mature neutrophils, G-CSF interacts with the G-CSF receptor leading to activation of the JAK-STAT signaling pathway, which controls the expression of the chemokine receptor CXCR2. CXCR2 is involved in promoting neutrophil release from the bone marrow compartment. Mammalian sterile 20-like kinase 1 (MST1) has been shown to be relevant in immune cell trafficking. Recently, increased numbers of neutrophils have been described in the bone marrow of *Mst1*-deficient mice suggesting a role of MST1 in regulating neutrophil homeostasis. Accordingly, I aimed to study the role of MST1 in neutrophil homeostasis including G-CSF receptor signaling and neutrophil mobilization.

In vivo intravenous injection of G-CSF into *Mst1*$^{-/-}$ mice failed to induce an increase in the circulating neutrophil pool 4 hours after injection when compared to wild type mice. Furthermore, using two-photon microscopy to *in vivo* image the bone marrow niche after G-CSF stimulation, I was able to observe a defect in the migratory behaviour of *Mst1*$^{-/-}$*Lyz2*^{eGFP} neutrophils and their decreased tendency of intravasating into the bone marrow microvasculature. Elucidating the molecular mechanisms of a putative regulation of G-CSF signaling by MST1, I investigated the expression and phosphorylation of G-CSF receptor and downstream signaling molecules using both flow cytometry and western blot. Interestingly, I detected diminished phosphorylation of STAT3 after *in vitro* G-CSF stimulation in *Mst1*$^{-/-}$ bone marrow derived neutrophils compared to wild type neutrophils. In addition, up-regulation of surface CXCR2 as a consequence of STAT3 transcriptional activity was also diminished in *Mst1*$^{-/-}$ neutrophils elucidating that MST1 regulates the G-CSF receptor-signaling cascade through STAT3 dependent transcriptional mechanisms. I finally found that after G-CSF stimulation *Mst1*$^{-/-}$ bone marrow

derived neutrophils displayed less STAT3 in its monomeric form than wild type neutrophils, revealing that mechanistically MST1 contributes to the G-CSF receptor signalling by promoting the intracellular availability of monomeric STAT3.

Taken together, I identify MST1 as a key player in G-CSF receptor signaling in bone marrow derived neutrophils and show that MST1 is critical for the proper mobilisation and release of mature neutrophils from the bone marrow into the blood circulation.

Table of contents

Figures and Tables

Figures

Tables

Abbreviation list

APC	Antigen presenting cell
BrdU	Bromodeoxyuridine
C/EBP	CCTA-enhancer-binding protein
CD	Cluster of differentiation
CID	Combined immunodeficiency
CRH domain	Cytokine receptor homology domain
CSF3	Colony stimulating factor 3
CSF3R	Colony stimulating factor 3 receptor
CXCR1	CXC chemokine receptor 1
CXCR2	CXC chemokine receptor 2
CXCR4	CXC chemokine receptor 4
CMP	Common myeloid progenitor
DC	Dendritic cell
DP thymocyte	Double positive thymocyte
ERK	Extracellular signal-regulated kinase
ESAM	Endothelial cell adhesion molecule
FOXP3	Forkhead box P3
G-CSF	Granulocyte colony-stimulating factor
G-CSFR	Granulocyte colony-stimulating factor receptor
GFI-1	Growth factor independent 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-CSFR	Granulocyte-macrophage colony-stimulating factor receptor
GMP	Granulocyte/macrophage progenitor
GVHD	Graft-versus-host disease
HoxB	Homebox B
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplant
HSPCs	Hematopoietic stem and progenitor cells
ICAM-1	Intercellular adhesion molecule 1
ICAM-2	Intercellular adhesion molecule 2
IL	Interleukin
JAK	Janus kinase
JAM	Junctional adhesion molecule
LATS	Large tumour suppressor
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
Mϕs	Macrophages
M-CSF	Macrophage colony-stimulating factor
Mac-1	Macrophage antigen 1

MEK/MAPKK	Mitogen-activated protein kinase-kinase
MHC	Major histocompatibility complex
MMP-9	Metalloproteinase 9
MPO	Myeloperoxidase
MPP	Multipotent progenitor
MST1	Mammalian Sterile 20-like kinase 1 (synonym: STK4)
mTOR	Mammalian target of rapamycin
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NFκB	Nuclear factor “kappa-light-chain-enhancer” of activated B cells
PECAM-1	Platelet endothelial cell adhesion molecule 1
PI3K	Phosphatidylinositol-3 kinase
PSGL-1	P-selectin glycoprotein ligand 1
PVR	Poliovirus receptor
RORγt	Retinoid acid related-orphan nuclear receptor γt
ROS	Reaxtive oxygen species
SARAH domain	Sav/Rassf/Hpo domain
SAV	Salvator
SCN	Severe congenital neutropenia
SDF-1	Stromal cell-derived factor 1
SH2 domain	Src homology 2 domain
SOCS3	Suppressor of cytokine signalling 3
SP thymocyte	Single positive thymocyte
STAT	Signal transducer and activator of transcription
STK4	Serine/threonine kinase 4 (synonym: MST1)
TAZ	Transcriptional co-activator with PDZ-binding motif
TCR	T cell receptor
TEM	Transendothelial migration
TGFβ	Transforming growth factor β
Th17	T helper 17 cell
TNFα	Tumour necrosis factor α
Treg	Regulatory T cell
TRIAL	TNFα-related apoptosis-inducing ligand
VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA	Very late antigen
YAP	Yes-associated protein

1. Introduction

1.1. The Neutrophil

Often neutrophils are referred as the infantry of our immune system since they are one of the most abundant leukocytes in circulation and are the first to be recruited when an insult occurs. Even though neutrophils represent 40-70% of circulating white blood cells in humans and 20% in mice, they are short-lived cells and therefore need to be continuously released from the bone marrow. It is estimated that human adults produce $2 \cdot 10^{11}$ neutrophils per day (Borregaard, 2010). Peripheral blood neutrophil numbers need to be tightly regulated, since neutropenia leads to infections and in some cases to death. Therefore, the synergy between neutrophil proliferation in the bone marrow, the release into the blood stream, and finally their clearance needs to be perfectly coordinated.

1.1.1. Neutrophil ontogeny and granulopoiesis

During vertebrates' development, ontogeny of the hematopoietic system comes in two waves. The first wave occurs from precursors located in the blood islands of the yolk sac and produces primitive erythrocytes and myeloid cells that migrate to the central nervous system and the skin to form the microglia and Langerhans cells (Medvinsky, Rybtsov and Taoudi, 2011). The second wave originates in the aorta-gonad-mesonephros (AGM) region of the dorsal aorta and gives rise to definitive haematopoiesis (Medvinsky, Rybtsov and Taoudi, 2011). Pre-hematopoietic stem cells (pre-HSCs) gradually migrate from the aorta to the liver and start to differentiate and expand to finally colonise the bone marrow (Golub and Cumano, 2013).

Adult haematopoiesis in the bone marrow has been widely studied and described. Haematopoiesis that leads to the production of neutrophils is also referred to as granulopoiesis. HSCs are undifferentiated cells and as definition have the ability of self-renewal and multipotency. HSCs differentiate into multipotent progenitors (MPPs), which retain the ability of multipotency but can no longer self renew. In mice, HSCs and MPPs compose the heterogeneous group of LSK cells, characterized by the phenotypic markers Lin$^-$, Sca-1$^+$, and c-Kit$^+$. In

humans, this multipotent group of cells are characterized for being Lin^-, $CD34^+$, and $CD38^-$ (Seita and Weissman, 2010). In the next differentiation step, progenitors are already lineage-committed. The one that will finally give rise to the neutrophil is the oligopotent common myeloid progenitor (CMP), which further differentiates to the granulocyte/macrophage progenitor (GMP) (Figure 1A).

From that stage on all neutrophil progenitors are unipotent and committed. The current definition of these progenitors has been based on morphological observations of hematopoietic stains under the microscope. In this classification, we find promyelocytes and myelocytes, which can both proliferate and differentiate, and finally metamyelocytes that can only evolve to band neutrophils and mature neutrophils (Figure 1B).

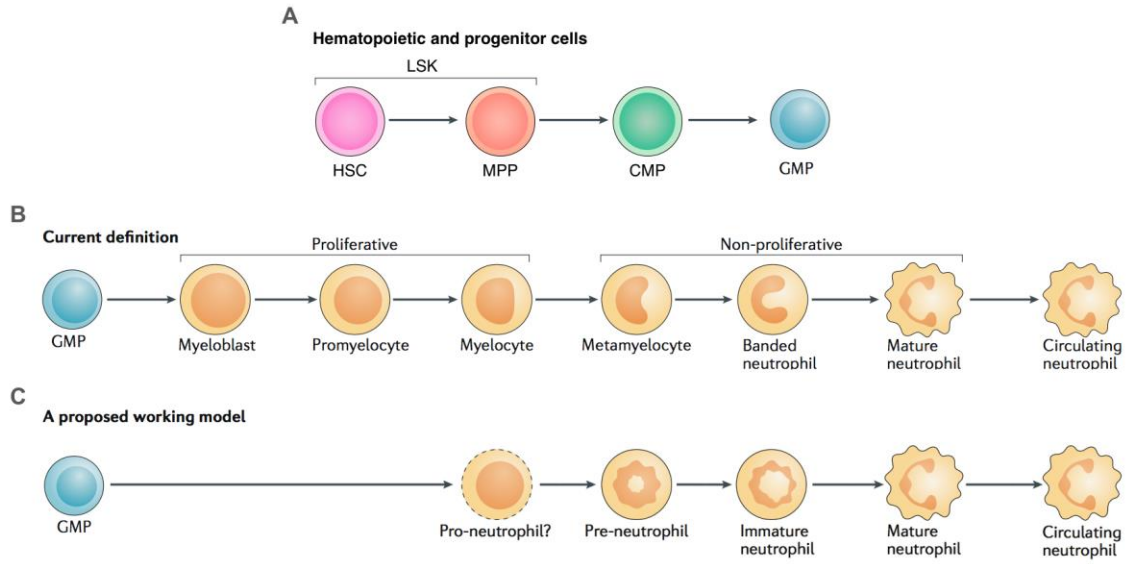

Figure 1. Granulopoiesis hierarchy. (**A**) Initial steps of granulopoiesis. (**B**) Neutrophil-committed progenitors as described by classical microscopy. (**C**) Proposed classification of neutrophil-committed progenitors based on CyTOF. *HSC – hematopoietic stem cell; MPP – multipotent progenitor; CMP – common myeloid progenitor; GMP – granulocyte/macrophage progenitor*. Modified from Ng, Ostuni and Hidalgo, 2019.

Recently an alternative and mass cytometry by time of flight (CyTOF) based classification of the pool of committed progenitors was published (Evrard *et al.*, 2018). In the publication, the investigators describe three subsets of neutrophils within the bone marrow: a committed proliferative neutrophil precursor (preNEU) that can differentiate into non-proliferative immature neutrophils and mature neutrophils (Figure 1C).

1.1.2. Regulation of granulopoiesis

Neutrophil differentiation is the combinatorial result of the expression of several key transcription factors, which can be influenced by cytokine signalling transmitting external signals. The expression of transcription factors GATA-2, Homebox B4 (HoxB4), Ikaros, and Notch1 promote HSCs self-renewal (Zhu and Emerson, 2002). On the other hand, the co-upregulation of PU.1 and GATA-1 in HSCs favours the differentiation to CMPs (Nerlov and Graf, 1998; Pierelli *et al.*, 2000). There is a physical interaction between PU.1 and GATA-1 that results in an inhibition of the transcription activity of the counterpart (Laslo *et al.*, 2008). The sustained expression of PU.1 and consequent repression of GATA-1 decants the differentiation towards the GMP. Otherwise, the expression of GATA-1 over PU.1 activity favours the progression towards the erytroid/megakaryocyte lineage. PU.1 is required for the proper expression of granulocyte-macrophage colony stimulating factor receptor (GM-CSFR) and macrophage colony stimulating factor receptor (M-CSFR), which signalling is necessary for the myeloid lineage commitment of CMPs and GMPs (Anderson *et al.*, 1998; DeKoter, Walsh and Singh, 1998). For terminal granulocytic differentiation, PU.1 expression is maintained and upregulation of CCTA-enhancer-binding protein α (C/EBPα) is required, which is a transcription factor that mediates transactivation of several genes, such as MPO and ELANE that code for primary granule components myeloperoxidase (MPO) and neutrophil elastase (NE) (Masgrau-Alsina, Sperandio and Rohwedder, 2020). The granulocyte colony stimulating factor receptor (G-CSFR) is also upregulated (Hohaus *et al.*, 1995) and even though it is not necessary for neutrophil commitment it appears to be fundamental for neutrophil maturation (Zhang *et al.*, 1997). At the same time, expression of transcriptional repressor growth factor independent 1 (GFI-1) is required for the repression of monocyte lineage genes such as M-CSF (Zarebski *et al.*, 2008). The expression of C/EBPε marks appearance of secondary granule components and the differentiation to myelocytes and metamyelocytes (Theilgaard-Mönch *et al.*, 2005). In the last stages of neutrophil maturation, transcription factors PU.1, C/EBPβ, C/EBPγ, and C/EBPδ increase and promote the formation of tertiary granules and secretory vesicles (Bjorregaard *et al.*, 2003; Fiedler and Brunner, 2012) (Figure 2).

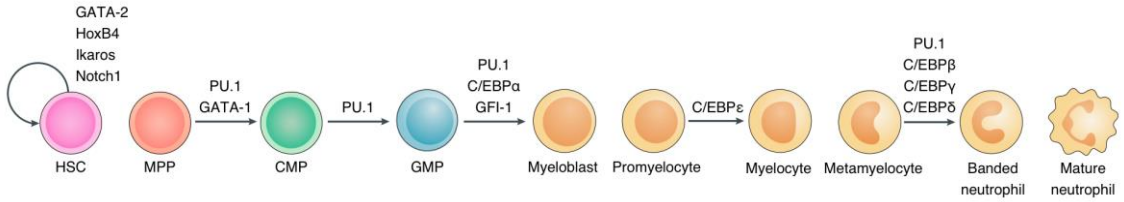

Figure 2. Key transcription factors regulating granulopoiesis. *HSC – hematopoietic stem cell; MPP – multipotent progenitor; CMP – common myeloid progenitor; GMP – granulocyte/macrophage progenitor; HoxB4 – Homebox B4; C/EBP – CCTA-enhancer binding protein; GFI-1 – growth factor independent 1*. Inspired by Zhu and Emerson, 2002; Fiedler and Brunner, 2012; and Ng, Ostuni and Hidalgo, 2019.

1.1.3. Chemokine receptors and chemokines in the neutrophil life cycle

Chemokine signalling mediates the release of freshly matured neutrophils from bone marrow to the circulation as well as the recruitment of senescent neutrophils to clearance organs. Chemokines are small proteins classified in four groups according to the spacing between two conserved cysteine residues at their N terminus: C, CC, CXC, and CX_3C. Chemokine receptors are cell surface receptors with seven-transmembrane domains and G-protein coupled. Neutrophils express mainly chemokine receptors that bind to CXC chemokines (Capucetti, Albano and Bonecchi, 2020).

CXC chemokine receptor 4 (CXCR4), also known as cluster of differentiation 184 (CD184), is highly expressed in HSCs and all neutrophil precursors, and it is downregulated when neutrophils reach complete maturation. The main ligand of CXCR4 is CXCL12, also known as stromal cell-derived factor 1 (SDF-1), which is constitutively and highly expressed by bone marrow stromal cells. CXCR4/CXCL12 interaction confers a strong and essential bone marrow retention signal. Mice with a myeloid lineage-specific deletion of CXCR4 present decreased numbers of bone marrow-resident neutrophils and higher numbers of circulating neutrophils (Eash *et al.*, 2009). Like other CXC chemokine receptors, CXCR4 interaction with its ligands promotes the receptor's clatrin-dependant endocytic internalization and depending on the duration of the chemokine stimulation the receptor-containing endosomes can undergo a recycling pathway or an ubiquitin-dependant degradation route (Borroni *et al.*, 2010). CXCR4 has also been proved to undergo constitutive internalization (Zhang *et al.*, 2004).

CXC chemokine receptor 2 (CXCR2) has a complete antagonistic function to CXCR4 in regard to neutrophil trafficking in the bone marrow. CXCR2 is upregulated in fully mature neutrophils and its signalling promotes neutrophil detachment and mobilisation to the circulation. It is quite a promiscuous receptor that in humans binds to CXCL1, 2, 3, 5, 6, 7, and 8 and CXCL1 (KC), 2 (MIP-2), and 5 in mice. The receptor-ligand interaction promotes the internalization of the receptor as well, but while CXCR4-containg endosomes can undergo rapid and slow recycling, CXCR2 can only follow the Rab11-dependant slow recycling route (Fan *et al.*, 2004; Masgrau-Alsina, Sperandio and Rohwedder, 2020). Activated CXCR2 promotes neutrophil migration through different G-protein-mediated signalling pathways, such as the phosphatidylinositol-3 kinase (PI3K)/Akt (Knall, Worthen and Johnson, 1997), the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) and the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (Nguyen-Jackson *et al.*, 2010). Moreover, CXCR2 signalling enhances the activity of nuclear factor "kappa-light-chain-enhancer" of activated B cells (NF-κB) that leads to the transcription of CXCR2 ligands CXCL1, 2, 3, and 8 (Cheng *et al.*, 2019) and therefore exercises a positive feedback loop. CXC chemokine receptor 1 (CXCR1) shares a 78% homology with CXCR2 and regulates neutrophil function in a very similar way. The few functional difference between the receptor are that CXCR1 can only bind to CXCL6 and 8 and has a slower internalization rate than CXCR2 (Cheng *et al.*, 2019).

CXCR4 downregulation and CXCR2 upregulation mark the culmination of neutrophil maturation and its release into the blood stream. If neutrophils are not recruited into inflamed tissue while patrolling the circulation, an inner senescent program is activated. This program starts with the downregulation of CXCR2, which blocks positive signalling for neutrophil migration, and the upregulation of CXCR4, that facilitates neutrophil homing to the bone marrow and other CXCL12 rich organs such as the spleen or the liver (Figure 3). In these clearance organs neutrophils become apoptotic and end up being phagocytosed by resident macrophages (Furze and Rankin, 2008). CXCR4/CXCL12 interaction in aged neutrophils has been claimed to promote neutrophil apoptosis through the up regulation of the cytokine TNFα-related apoptosis-inducing ligand (TRIAL), which

acts in an autocrine or paracrine way on the as well upregulated death-inducing TRAIL receptor (Lum *et al.*, 2005).

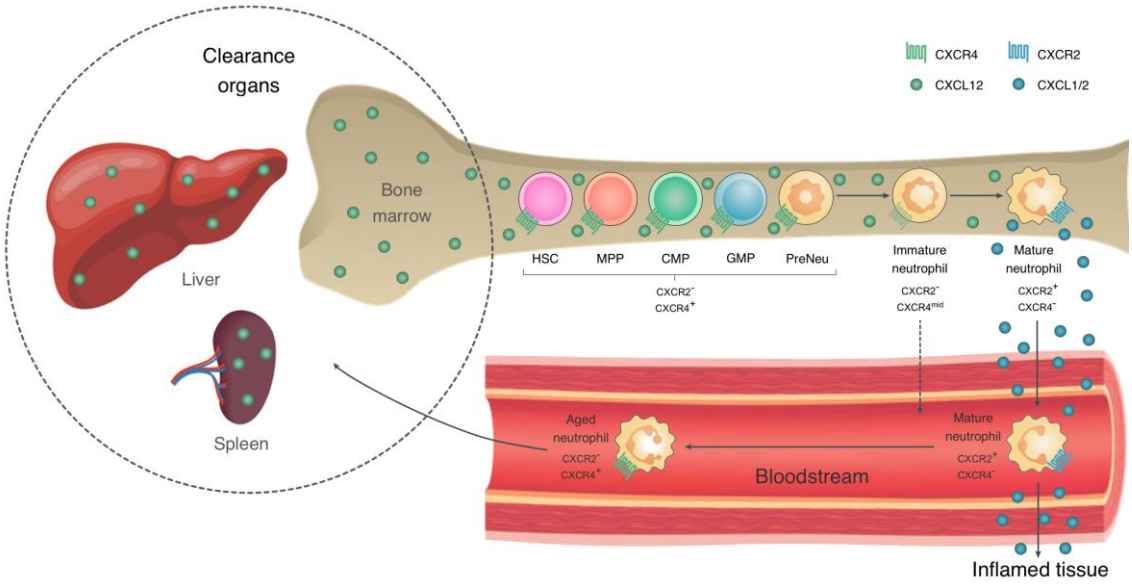

Figure 3. CXCR4 and CXCR2 in the neutrophil life cycle. Neutrophil progenitors CXCR4 expression provides a strong retaining signal to the CXCL12-rich bone marrow. Neutrophil maturation culminates with the downregulation of CXCR4 and upregulation of CXCR2, favouring neutrophil release to the circulation. Aged neutrophils switch again their CXCR2/4 expression pattern leading to trafficking into the bone marrow and other CXCL12 rich organs to get cleared. *HSC – hematopoietic stem cell; MPP – multipotent progenitor; CMP – common myeloid progenitor; GMP – granulocyte/macrophage progenitor*.

1.1.4. Neutrophil recruitment cascade

An inflammatory process is crucial to clear an injury and re-establish homeostasis, but it needs to be a controlled process and limited in time and extension since a perpetuation of inflammation can lead to severe problems for the organism. Therefore, free-flowing neutrophils should only get activated at required places. Neutrophil activation and the subsequent recruitment into the inflamed tissue require a series of sequential molecule expression and distribution changes in the leukocyte and the endothelium that is also known as the neutrophil recruitment cascade.

When the insult occurs, tissue resident cells such as macrophages release inflammatory mediators that induce the mobilization of endothelial P-selectin to the cell membrane that interacts with constitutively expressed P-selectin glycoprotein ligand 1 (PSGL-1) on neutrophils. This interaction stimulates

neutrophil rolling on the endothelium and favours the high affinity state of neutrophil β_2 integrins lymphocyte function-associated antigen 1 (LFA-1) and macrophage antigen 1 (Mac-1). The signalling provided by the interaction of neutrophil CXCR2 with endothelial CXCL1 contributes as well to integrin activation (Sawant *et al.*, 2016).

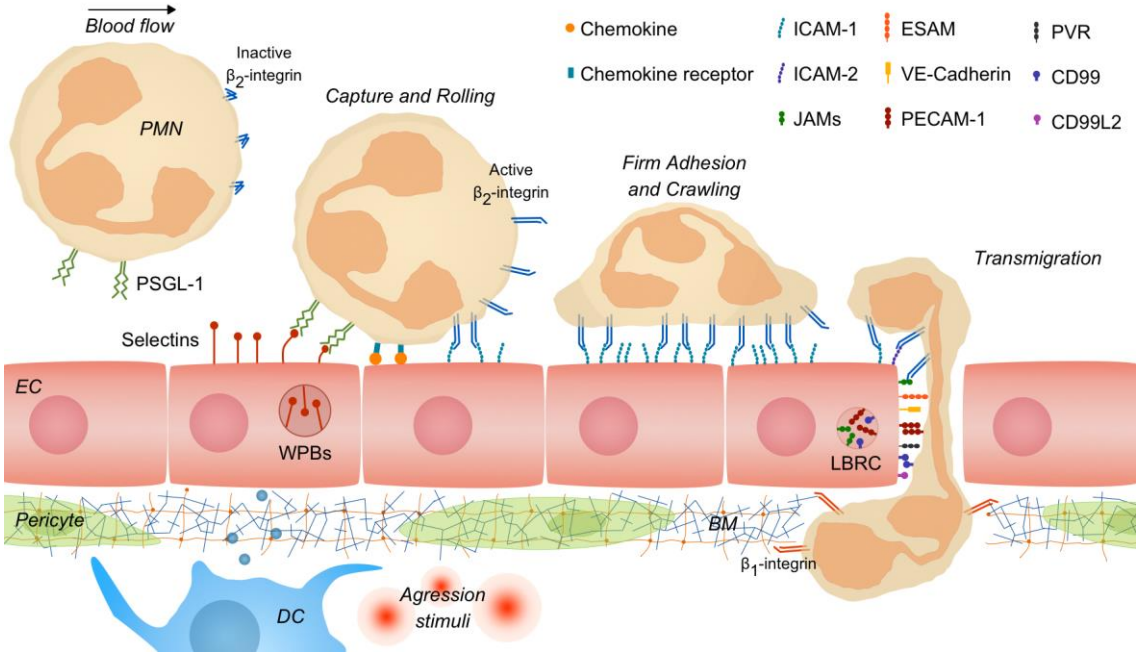

Figure 4. Neutrophil recruitment cascade. Neutrophils recognize selectins on the inflamed endothelium and interact with endothelial chemokines. Activation of neutrophil β_2 integrins provide firm adhesion and crawling by interaction with endothelial ICAM-1. This interaction facilitates the later transmigration of the neutrophil to finally reach the inflamed area. *PSGL-1 – P-selectin glycoprotein ligand 1; WPBs – Weibel-Palade bodies; LBRC – lateral border recycling compartment; ICAM – intracellular adhesion molecule; JAMs – junctional adhesion molecules; ESAM – endothelial cell adhesion molecule; PECAM-1 – platelet endothelial cell adhesion molecule; PVR – poliovirus receptor.*

Integrins act as bidirectional mediators in signal transduction. By inside-out signalling, integrins acquire a high affinity conformation that favours ligand binding, and at the same time ligand binding triggers outside-in signalling that leads to cell spreading and migration. β_2 integrins establish firm bonds with endothelial intracellular adhesion molecule 1 (ICAM-1), which favour a firm adhesion of neutrophils. ICAM-1 activates a Ca^{2+} dependent signalling in the endothelial cell that cause actin cytoskeleton rearrangements and mobilisation of several molecules that facilitate neutrophil transendothelial migration (TEM) in a

sequential manner such as ICAM-1, intracellular adhesion molecule 2 (ICAM-2), junctional adhesion molecules (JAMs), endothelial cell adhesion molecule (ESAM), platelet endothelial cell adhesion molecule 1 (PECAM-1), poliovirus receptor (PVR), CD99, and CD99L2. In the final steps of recruitment, β_1 integrins very late antigen-3 (VLA-3) and very late antigen-6 (VLA-6), and NE have been proven indispensable for the neutrophil to penetrate the basement membrane, the last barrier to be crossed before reaching the site of inflammation (Masgrau-Alsina, Sperandio and Rohwedder, 2020) (Figure 4).

1.1.5. Neutrophil methods of action

Once at sites of inflammation, neutrophils have several ways to fight against pathogens including phagocytosis, degranulation, and NETosis.

1.1.5.1. Phagocytosis

Phagocytosis is the physical uptake of debris, microorganisms or dead cells and its encapsulation in an intracellular compartment called phagosome. Neutrophils share this ability with monocytes, macrophages, mast cells and dendritic cells. The final purpose of phagocytes is to remove pathogens. For this process, reactive oxygen species (ROS) production by NAPDH oxidase is indispensable to fight the microbes (Roos, 2019). NADPH oxidase is a protein complex present in the phagosome membrane and composed by two transmembrane subunits, three cytosolic subunits and a Rac small GTPase. In the resting state, the different subunits are physically separated, but after the neutrophil activation the cytosolic subunits are phosphorylated and able to interact with the transmembrane subunits, allowing the complex to function and release ROS inside the phagosome (Nguyen, Green and Mecsas, 2017).

1.1.5.2. Degranulation

Neutrophils, together with eosinophils and basophils, compose the group of granulocytes. This name refers to the high content of granules they have in their cytoplasm. More than being a characteristic trait, those granules are of main importance for the neutrophil's proper function. There are four types of granules classified according to their appearance in the different stages of maturation: primary or azurophilic (myeloblasts and promyelocytes), secondary or specific

(myelocytes and metamyelocytes), tertiary or gelatinase (band neutrophils) and secretory vesicles (segmented cells) (Masgrau-Alsina, Sperandio and Rohwedder, 2020). Curiously, during degranulation to the extracellular space, granules are released in a sequential fashion (in the inversed order they appear during development). Secretory vesicles, mainly containing adhesion molecules, are first discharged followed by gelatinase granules, specific granules and finally azurophilic granules. The last two are the ones containing the highest antimicrobial content and apart from being released also fuse with phagosomes (Nordenfelt and Tapper, 2010).

1.1.5.3. Neutrophil extracellular traps (NETs)

Highly activated neutrophils can release (but remain attached) a net-like structure consisting of a DNA scaffold associated with histones and granular proteins called neutrophil extracellular trap (NET), in a process denominated NETosis. The main purpose of NETs is to retain pathogens and physically prevent them to damage the organism. Different associated granular proteins can contribute to direct microbial killing with their antimicrobial properties (lactoferrin, defensins, LL37, bacterial permeability increasing protein), protease-like action (NE, proteinase 3, cathepsin G, gelatinase) and ROS production (MPO).

1.2. Granulocyte colony-stimulating factor (G-CSF)

Back in the 1960s, the discovery of the first cytokines enlightened the research of cell signalling between immune cells. Cytokines are small peptides secreted by immune cells (macrophages, B cells, T cells and mast cells) but also by non-immune cells such as endothelial cells, fibroblasts and stromal cells. Under normal conditions, cytokines are found in low concentration in serum but levels dramatically increase in an inflammatory environment and go back to normal after recovery (Watari *et al.*, 1989).

The granulocyte colony-stimulating factor (G-CSF) or colony stimulating factor 3 (CSF3) was one of the first cytokines to be identified and it probably has been the widest therapeutically used cytokine up to date. Human G-CSF is a 18.8kDa protein with 175 amino acids that was first cloned in 1986 (Souza *et al.*, 1986). Ever since, it has been used to increase neutrophil numbers in cancer patients and for the mobilisation of peripheral stem cells.

1.2.1. Control of G-CSF production by IL-23/IL-17 axis

G-CSF is the major cytokine regulating neutrophil mobilisation to the circulation and therefore G-CSF levels are tightly regulated by a complex mechanism involving the interleukin (IL)-23/IL-17 axis and T_H17 cells. Naïve $CD4^+$ T cells can differentiate into T helper 17 cells (Th17) or regulatory T cells (Treg) among other regulatory and effector T cell subsets (Bunte and Beikler, 2019). The production of pro-inflammatory Th17 and anti-inflammatory Treg cells is maintained in equilibrium. Transforming growth factor β (TGFβ) initiates the differentiation of naïve $CD4^+$ T cells into Treg cells by activating forkhead box P3 (FOXP3) via STAT6. If at the same time the cell is stimulated by IL-6 in mice and IL-1β in humans, FOXP3 is repressed and retinoid acid related-orphan nuclear receptor γt (RORγt) is activated via STAT3 (Figure 5). RORγt promotes the expression of IL-17 and IL-23 receptor (IL-23R). Tissue resident antigen presenting cells such as dendritic cells (DCs) and macrophages (MΦs) produce IL-23, that promote Th17 maintenance and expansion, and boosts the production of RORγt and IL-17 (Nurieva *et al.*, 2007). IL-17 receptor is expressed in endothelial cells, fibroblasts, macrophages and other cell types (Iwakura and Ishigame, 2006).

In those cells, IL-17 stimulates the production of CXC cytokine receptor ligands (CXCL1, 2, 5 and 8) and induces G-CSF production via C/EBPβ and NFκB (Veldhoen, 2017) that promote the differentiation and release of neutrophils to the circulation. Phagocytosis of apoptotic neutrophils in tissue by DCs and MΦs curbs the secretion of IL-23 and thus negatively regulates the IL-23/IL-17 axis to control the number of neutrophils in the circulation (Stark *et al.*, 2005) (Figure 5).

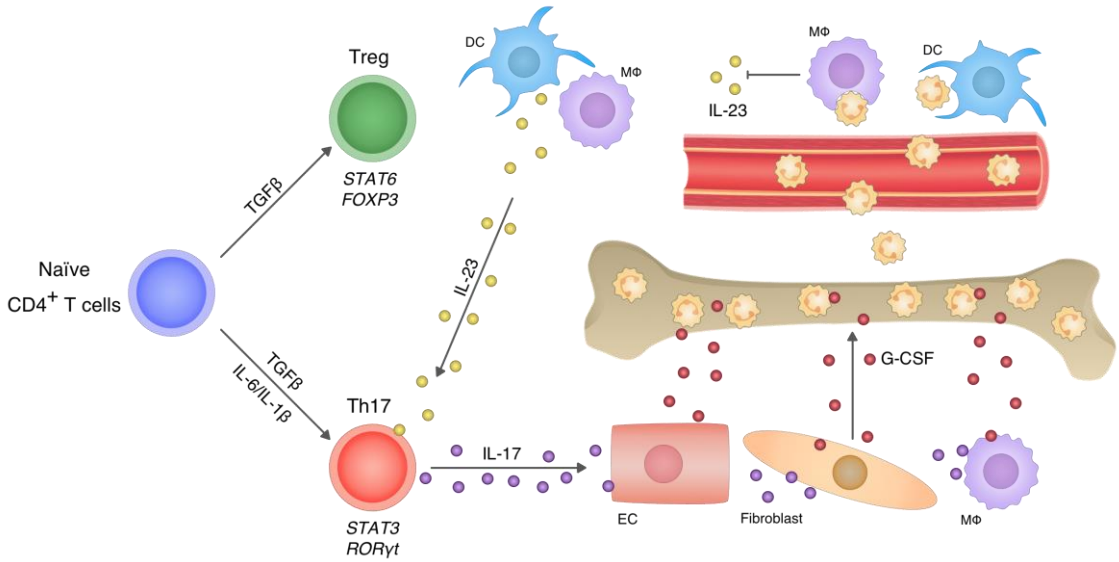

Figure 5. IL-23/IL-17/G-CSF axis. TGFβ and IL-6/IL-1β control the equilibrium between regulatory T cells (Treg) and T helper 17 cells (Th17). IL-23 enhances IL-17 production by Th17 cells and IL-17 stimulate endothelial cells, fibroblasts and macrophages to release G-CSF and promote neutrophil mobilisation to the circulation. Neutrophil phagocytosis by resident phagocytes curb IL-23 production and therefore negative regulate the IL-23/IL-17/G-CSF axis. *TGFβ – transforming growth factor β; IL – interleukin; STAT – signal transducer and activator of transcription; FOXP3 – forkhead box P3; G-CSF – granulocyte colony stimulating factor; EC – endothelial cell; MΦ – macrophage; DC – dendritic cell.*

G-CSF production can also be stimulated in response to vascular endothelial growth factor (VEGF), interleukin 1β (IL-1β), lipopolysaccharide (LPS) and tumour necrosis factor α (TNFα) (Theyab *et al.*, 2021).

1.2.2. G-CSF receptor (G-CSFR)

The granulocyte colony-stimulating factor receptor (G-CSFR), also known as colony stimulating factor 3 receptor (CSF3R) or cluster of differentiation 114 (CD114) is a 813 amino acid transmembrane protein encoded by the *CSF3R* gene and exclusively expressed on the membrane of myeloid cells and the placenta. Its extracellular domain is comprised by an immunoglobulin-like (Ig-like) domain, a cytokine receptor homology (CRH) domain, a WSXWS motif, and three fibronectin type III (FN III) domains (Fukunaga *et al.*, 1991) (Figure 6). Upon G-CSF binding to G-CSFR's CHR domain, the receptor undergoes conformational changes that induce the dimerization of the receptor-ligand complex in a 2:2 stoichiometry leading to the activation of the receptor (Horan *et al.*, 1996; Layton and Hall, 2006). The cytoplasmic tail of the receptor is divided into three boxes (Box1-3) and has four conserved tyrosine residues (704, 729, 744 and 764) that provide docking sites for Src homology 2 (SH2)-domain containing proteins that trigger downstream signalling of the receptor (Fukunaga *et al.*, 1991).

G-CSFR does not have an intrinsic tyrosine kinase activity but its homodimeric form activates several substrates such as JAK1, JAK2, TYK2, SRC family kinases, SYK and TNK (Corey *et al.*, 1994; Avalos *et al.*, 1997; Dwivedi and Greis, 2017). Of those, the JAK2 seems to have a predominant role. JAK2 interaction with the SH2 domain of the dimerized receptor brings two JAK2 molecules to close proximity and favours its activation by trans-phosphorylation (Theyab *et al.*, 2021) (Figure 6). Subsequently, it phosphorylates (1) the G-CSF receptor at the aforementioned tyrosine residues and (2) STAT proteins. Phosphorylated tyrosine residues 704 and 744 of the G-CSFR have been described to serve as indispensable docking sites for STAT3 to favour its phosphorylation by JAK2 (Ward *et al.*, 1999) (Figure 6). STAT proteins are the major substrates of JAK kinases, and G-CSF signalling activates principally STAT3 but also STAT1 and STAT5 (Tian *et al.*, 1996).

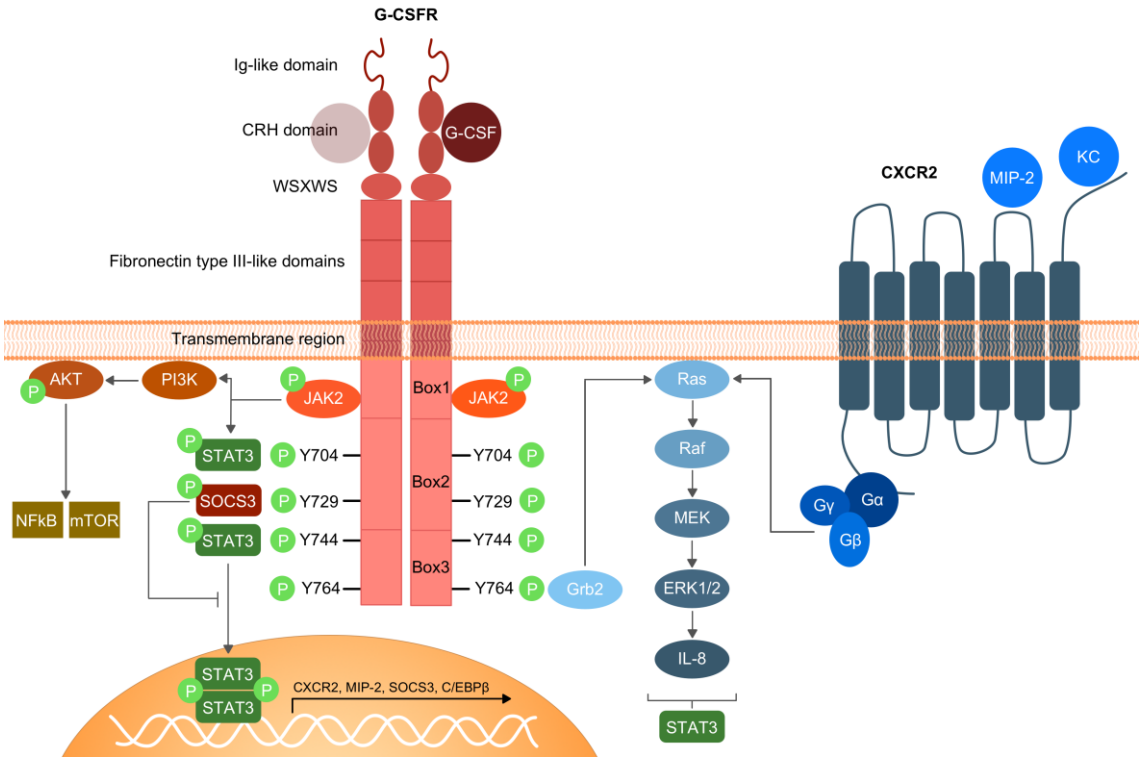

Figure 6. G-CSFR signalling. G-CSF interaction with the CRH domain of the receptor induces structural changes that favour receptor homodimerization and changes in the cytoplasmic tail leading to the activation of several signalling pathways. JAK2 has a predominant role by activating the PI3K/Akt pathway to promote proliferation and STAT3 that promotes cell mobilization and granulopoiesis, as well as the transcription of SOCS3 that negatively regulates the G-CSFR signalling. Moreover, G-CSFR signalling modulates the amplitude of the MEK/ERK pathway triggered by CXCR2, which also favours neutrophil mobilization. *G-CSF – granulocyte colony stimulating factor; G-CSFR – GCSF receptor; JAK2 – Janus kinase 2; STAT3 – signal transduces and activator of transcription 3; SOCS3 – suppressor of cytokine signalling 3; PI3K – phosphoinositide-3 kinase; NFκB – nuclear factor "kappa-light-chain-enhancer" of activated B-cells; mTOR – mammalian target of rapamycin; Ras – Rat sarcoma virus; MEK – mitogen-activated protein kinase kinase; ERK – extracellular signal regulated kinase; IL-8 – interleukin 8.*

Engagement of the G-CSFR also activates LYN, the most predominantly expressed Src kinase in phagocytes. Synergically with JAK2, LYN activates the PI3K/Akt pathway, which promotes cell proliferation (Polak and Buitenhuis, 2012) and contributes to block apoptosis by NFκB and mammalian target of rapamycin (mTOR). Moreover, the PI3K/Akt pathway stimulates the phosphorylation and membrane translocation of NAPDH oxidase subunit $p47^{phox}$ and therefore controls ROS production, essential for neutrophil defence against pathogens (Zhu *et al.*, 2006). Other G-CSFR proliferative signals are transmitted by the activation of the

MAPK/ERK signalling pathway through the intermediate proteins Shc and Grb2 that activates Ras (Kendrick and Bogoyevitch, 2007).

G-CSF stimulation has been correlated with elevated levels of metalloproteinase 9 (MMP-9), NE, and cathepsin G in the bone marrow. These proteases cleave surface proteins that keep neutrophils and its progenitors attached to the bone marrow niche, such as CXCL12 and its receptor CXCR4, c-KitL (c-Kit ligand), and VCAM-1 (VLA-4 ligand). Serine protease inhibitors (serpins) levels are also reduced after G-CSF stimulation (Greenbaum and Link, 2011). The release of these proteinases contributes to neutrophil and hematopoietic stem and progenitor cell (HSPC) mobilisation but is not required, since mice lacking combinations of MMP-9, NE and cathepsin G show normal mobilisation in response to G-CSF or IL-8 (Levesque *et al.*, 2004).

As observed with other cytokine receptors, after binding its ligand the G-CSFR gets internalized. Through the action of lysine ubiquitine ligases and protein kinases the receptor can be directed to lysosomes for degradation or recycled to the plasma membrane passing towards the endoplasmic reticulum and the Golgi apparatus (Dwivedi and Greis, 2017). Compared to the total intracellular pool only a small fraction of G-CSFR is expressed on the plasma membrane (Palande *et al.*, 2013) and most of the intracellular G-CSFR is associated with the Golgi or the lysosome. The exact mechanism that modulates the G-CSFR fate has not been described yet. As observed in cell line models, high expression of JAK kinases associate with C-CSFR and protect the receptor from degradation (Barge *et al.*, 1996). Finally, E3 ubiquitin ligase activity of suppressor of cytokine signalling 3 (SOCS3), which expression is directly controlled by STAT3, has shown to ubiquitinylate G-CSFR on lysine residue K632 affecting the trafficking of G-CSFR from early to late endosomes (Irandoust *et al.*, 2007).

1.2.3. STAT3 in the G-CSFR signalling pathway

1.2.3.1. STAT3 and granulopoiesis

G-CSF is the major cytokine coordinating neutrophil differentiation from hematopoietic stem cells (HSCs) in both the steady state and in emergency granulopoiesis. STAT3 contributes to this G-CSF-dependent granulopoiesis by

inducing the expression of CCAT-enhancer-binding protein β (C/EBPβ), a transcription factor that binds to the promoter site of *c-myc* and therefore stimulates the proliferation and differentiation of myeloid cells (Zhang *et al.*, 2010). Of note, STAT3 is totally dispensable for the proper differentiation of neutrophils. Actually, STAT3 bone marrow conditional knockout mice do not only show normal neutrophil maturation but also present peripheral neutrophilia in the steady state and enhanced maturation in emergency granulopoiesis (Lee *et al.*, 2002). STAT3 has a major role in the G-CSFR signalling pathway and also regulates a negative feedback loop to control neutrophil numbers by promoting the expression of suppressor of cytokine signalling 3 (SOCS3), a member of the STAT-induced STAT inhibitors (SSI) that as its name indicates blocks the G-CSFR signalling at different levels. For instance, SOCS3 binds to the phosphorylated tyrosine residue 729 of activated G-CSFR to induce the ubiquitination and consequent proteasome-mediated degradation of the receptor (Palande *et al.*, 2013). SOCS3 also has a central SH2 domain through which it can directly bind phospho-JAKs and inhibit their kinase activity (Carow and Rottenberg, 2014). SOCS3 has also been described to directly inhibit the activation of STAT3, probably also through its SH2 domain (Yasukawa *et al.*, 2003).

1.2.3.2. STAT3 and neutrophil mobilisation

G-CSF itself is not chemotactic (Wengner *et al.*, 2008). Even though, through STAT3 activation G-CSF moderates CXCR2 response to their ligands CXCL1 (KC) and CXCL2 (MIP-2). In fact, CXCR2 is much more potent for neutrophil mobilisation than G-CSF stimulation (Pelus and Fukuda, 2006). CXCR2 signalling activates the Raf/MEK/ERK pathway that favours neutrophil mobilisation by IL-8 production. Nguyen-Jackson et al. demonstrated that G-CSFR-activated STAT3 favours neutrophil mobilisation by (1) modulating the amplitude of the CXCR2-triggered Raf/MEK/ERK pathway and (2) directly enhancing the transcription of genes *Il8rb* and *Cxcl2* that code for CXCR2 and its ligand CXCL2, respectively, and therefore boosting the signalling pathway (Zhang *et al.*, 2010; Nguyen-Jackson *et al.*, 2012).

1.2.3.3. STAT protein signalling

In the classical paradigm of STAT-signalling, cytokine-free cells had monomeric STAT molecules floating in the cytoplasm and only upon cytokine stimulation, STAT molecules were phosphorylated by JAK kinases and acquired the ability to form dimers and migrate to the nucleus to act as transcription factors. In the late 90s, the paradigm changed after a report demonstrated that few STAT3, STAT1, STAT5a, and STAT5b molecules existed in the cytoplasm in a monomeric form. Instead, they were being part of complexes of 200-400kDa and 1-2MDa named statosome I and statosome II respectively. As shown, statosome I consisted of a STAT molecule associated with other polypeptides that include regulatory proteins, chaperones, membrane trafficking proteins, mitochondria proteins, and nuclear trafficking proteins (Ndubuisi *et al.*, 1999; Sehgal, 2008; Unudurthi *et al.*, 2018). It has also been shown that un-phosphorylated STAT proteins shuttle in a constitutive manner from the cytoplasm to the nucleus and vice versa (Liu, McBride and Reich, 2005), and that un-phosphorylated STATs can also induce transcription of genes, even though different sets than phosphorylated STATs (Yang *et al.*, 2005). Finally, the belief that after stimulation STAT proteins trafficking to the nucleus was a free cytosolic process has also changed, since it was proved that al least a portion of STAT proteins associate to calveolar and endocytic pathways (Shah *et al.*, 2006).

1.3. Mammalian Sterile 20-like kinase 1 (MST1)

Mammalian sterile 20-like kinase 1 (MST1) or serine/threonine kinase 4 (STK4) is a 487 amino acid long protein constitutively expressed in all mammalian cells. The structure of MST1 consists of a serine/threonine kinase domain at the N-teminus, a coiled coil region, and a C-terminal Sav/Rassf/Hpo (SARAH) domain (Kurz, Catz and Sperandio, 2018) (Figure 7). Through the SARAH domain MST1 interacts with its ligands and forms antiparallel dimers. After dimer formation MST1 gets activated by trans-autophosphorylation of threonine residue 183. In response to apoptotic stimuli, MST1 can be cleaved by caspase 3 at residues 326 (mice and humans) and 349 (humans) that separate the catalytic from the SARAH domain (Kurz, Catz and Sperandio, 2018).

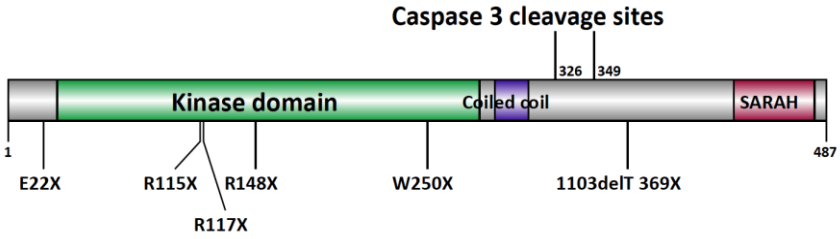

Figure 7. MST1 structure. MST1 has three main domains: the kinase domain (green), a coiled coil (purple) and a SARAH domain (pink). MST1 can be cleaved by caspase 3 at residues 326 and 349. Figure from Kurz, Catz and Sperandio, 2018.

MST1 belongs to the family of MST kinases that are highly conserved in all metazoans (Creasy and Chernoff, 1995). Of all paralogues (MST1-5), MST1 has the greatest homology to MST2, sharing 88% similarity and 76% identity of amino acid sequence. MST1/2 are >95% similar in their catalytic domains, indicating redundant functions. In fact, even though with a lower affinity to homodimers, MST1/2 establish functional heterodimers. While MST1/2 double knockout mice suffer embryonic lethality at day E8.5, MST1 or MST2 single knockout murine strains are viable (Oh *et al.*, 2009). *Mst2*$^{-/-}$ mice barely show any phenotype. In contrast, *Mst1*$^{-/-}$ mice exhibit a strong phenotype that will be exposed in the following chapters.

1.3.1. MST1 canonical signalling pathway in development

The MST1 canonical signalling pathway is related to organ growth and proliferation and is highly conserved in evolution (Pan, 2010). It was first described in *Drosophila melanogaster*, where the MST1 ortholog receive the name of Hippo. Hippo discovery occurred during a genetic screening in flies to find tumour suppressor genes. Loss of function mutations of the Hippo gene and other genes originated a tissue-overgrowth phenotype with increased proliferation and diminished apoptosis (Reddy and Irvine, 2008). The investigators named the protein Hippo because the phenotype they observed reminded them of a hippopotamus shape (Figure 8A-D). In this thesis work, I will use the mammalian nomenclature. The homolog proteins of the canonical pathway in *Drosophila melanogaster* can be found in Table 1.

Mammals	Drosophila melanogaster
MST1/2	Hippo (Hpo)
SAV1	Salvador (Sav)
MOB1	Mob
LATS1/2	Warts (Wts)
YAP/TAZ	Yorkie (Yki)
TEAD	Scalloped (Sd)

Table 1. MST1 canonical pathway nomenclature.

Upon activation, MST1 forms heterodimers with salvador homolog 1 (SAV1) and two MST1-SAV1 heterodimers further dimerize (Bae *et al.*, 2017). This scaffolding brings together two MST1 proteins that get trans-phosphorylated and also phosphorylate SAV1, MOB1, and large tumour suppressor kinases 1 and 2 (LATS1/2). LATS1/2 phosphorylate Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), which are then sequestered in the cytoplasm by binding to 14-3-3. YAP/TAZ are transcription co-activators that do not contain DNA binding domains *per se*, but interact with DNA-binding transcription factors such as TEAD family (TEAD1-4) in the nucleus and regulate several genes related with proliferation and survival (Figure 8F).

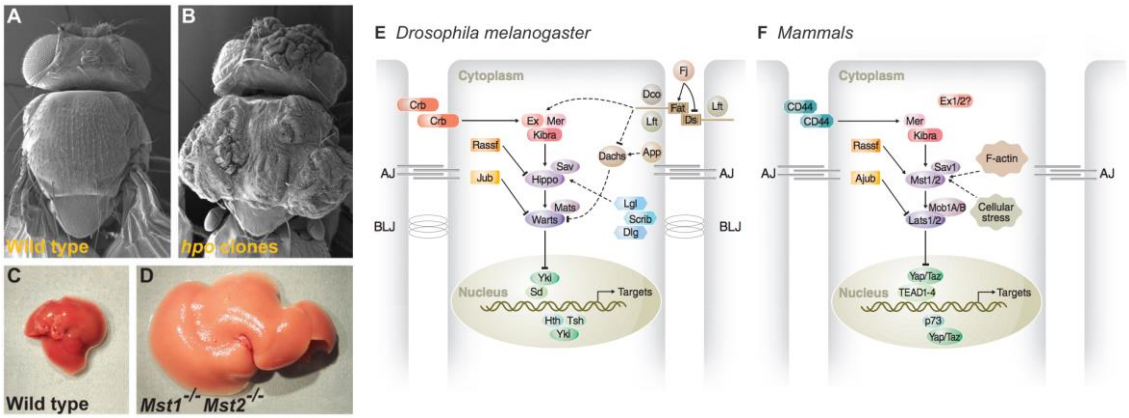

Figure 8. Phenotype of Hippo/MST1 mutants and canonical pathway in *Drosophila melanogaster* and mammals. (**A**) Normal fly cuticle in a wild type adult. (**B**) Overgrown fly cuticle in a *hpo*$^{-/-}$ *individual*. (**C**) Wild type mouse liver. (**D**) *Mst1/2*$^{-/-}$ mouse liver. (**E-F**) Scheme of the pathway in flies and mammals. Figures from Halder and Johnson, 2011.

1.3.2. MST1 and leukocyte function through the non-canonical signalling pathway

In 2012, two independent groups attributed the cause of a combined immunodeficiency (CID) in three unrelated consanguine families to biallelic loss-of-function mutations in *Mst1* (Abdollahpour *et al.*, 2012; Nehme *et al.*, 2012). These articles and the case study reports that followed (Crequer *et al.*, 2012; Halacli *et al.*, 2015; Dang *et al.*, 2016; Lum *et al.*, 2016; Schipp *et al.*, 2018; Al-Saud *et al.*, 2019; Cagdas *et al.*, 2021) demonstrated that MST1 is of major importance for proper leukocyte function. The main characteristics of human MST1-deficiency are T-and B-cell lymphopenia and transient neutropenia (Abdollahpour *et al.*, 2012). In most cases, symptoms appear in the first decade of life (Cagdas *et al.*, 2021) and generally include recurrent bacterial infections, viral infections, mucocutaneous candidiasis, atopy, cutaneous warts, and autoimmune manifestations (Abdollahpour *et al.*, 2012; Cagdas *et al.*, 2021). No dysmorphic symptoms were described in any of the patients (Nehme *et al.*, 2012). Treatment of those patients include immunoglobulins and prophylactic antibiotics. It is still unclear if hematopoietic stem cell transplantation (HSCT) is a suitable treatment for this syndrome since only 4 out of 8 (50%) MST1-deficient patients (from a cohort of 24 patients) that were reported to undergo the procedure survived, a lower rate than in other CIDs (Cagdas *et al.*, 2021). In the report by Nehme et al.

2012, 2 out of 3 siblings died due to graf-versus-host disease (GVHD) after undergoing HSCT but the successful transplant lead to the resolution of the immunodeficiency (Nehme *et al.*, 2012).

1.3.2.1. T cell development and function

T cell progenitors migrate from the bone marrow to the thymus, where they go through different stages of development marked by the expression of CD4 and CD8. Initially all cells are double negative (DN, $CD4^-CD8^-$) and after the successful reorganization of the β chain of the T-cell receptor (TCR) they become double positive (DP, $CD4^+CD8^+$). DP cells go through a process of positive selection, where thymocytes are exposed to self-antigens in the cortex by major histocompatibility complexes (MHC) on the surface of antigen presenting cells (APC), and those which are at least weakly able to bind MHC become single positive (SP, $CD4^-CD8^+$ if they interacted with MHC class I and $CD4^+CD8^-$ if they interacted with MHC class II). After that, SP thymocytes migrate to the medulla and undergo a negative selection, where cells with enhanced affinity to MHC molecules are destroyed to avoid self-harm and establish central tolerance. The resulting cells are naïve T cells that are ready to migrate to the periphery (Klein *et al.*, 2014). The dramatic decrease on T cell number reported by different studies on MST1-deficient patients (Zhou *et al.*, 2008; Choi *et al.*, 2009; Dong *et al.*, 2009; Katagiri *et al.*, 2009) as well as in murine studies (Kurz *et al.*, 2016a) contrasts with the increased numbers of SP thymocytes in *Mst1*$^{-/-}$ mice (Dong *et al.*, 2009; Katagiri *et al.*, 2009). Mou and others described that in *Mst1/2*$^{-/-}$ mice SP cell egress in the thymus was reduced by more than 90% with an increase in apoptosis (Mou *et al.*, 2012a). Moreover, it was reported that *Mst1*$^{-/-}$ had inefficient LFA-1/ICAM-1 mediated T cell adhesion within the medulla impairing the negative selection and resulting in defective antigen recognition by $CD4^+$ cells (Ueda *et al.*, 2012).

Further studies on LFA-1-mediated adhesion of T cells revealed the importance of MST1 in the reorganization of integrins and clustering to the leading edge of migrating cells. LFA-1 molecules are constitutionally expressed in lymphocytes in a low affinity state and to enhance the integrin affinity the α_L and the β_2 domains need to be separated (Cheng *et al.*, 2018). MST1 contributes to LFA-1 affinity maturation by forming a complex with RIAM and Talin (Kliche *et al.*,

2012). After chemokine stimulation, Rap1 binds RAPL, which at the same time interact and activate MST1 and form a complex with the α_L subunit of LFA-1 (Katagiri, Imamura and Kinashi, 2006). Moreover, MST1 phosphorylates and activates Rab13 effector DENND1C, and therefore promotes the translocation of LFA-1-containing vesicles to the cell membrane along the actin filaments in a myosin-Va-dependent manner (Nishikimi *et al.*, 2014) (Figure 9A). MST1 further impacts Rab13 vesicle trafficking by phosphorylating vasodilator-stimulated phosphoprotein (VASP) that regulates F-actin polymerization (Nishikimi *et al.*, 2014).

1.3.2.2. Treg/Th17 balance

Naïve T $CD4^+$ cells follow different paths of differentiation depending on the cytokine environment and can result in T helper 1 (Th1), Th2, Th17, or Treg cells (Afzali *et al.*, 2007). Treg cells have immunosupressor characteristics while Th17 cells promote inflammation through IL-17 production (Figure 5). Activation of TAZ via SMAD3 and STAT3 after TGFβ and IL-6/IL-26 simultaneous stimulation is indispensable for the differentiation to a Treg cell, since TAZ (1) is a critical co-activator of RORγt and (2) decreases acetylation of pro-Th17 cell FOXP3 (Geng *et al.*, 2017) (Figure 9B). MST1 is a suppressor of TAZ (Figure 8F) and therefore MST1 absence results in a higher activation of TAZ and the enhanced production of Th17 cells detrimental to Treg production. In mice, MST1 absence produces decreased levels of Treg cells during infancy that are restored in adulthood, and the phenotype is aggravated in *Mst1/2*$^{-/-}$ mice (Du *et al.*, 2014). This imbalance may contribute to the rise of autoimmune and inflammatory diseases observed in MST1-deficient patients (Geng *et al.*, 2017).

Interestingly, dendritic cells' MST1 also affects naïve T cell differentiation towards Th17 cells. MST1 deficiency activates p38 MAPK signal on DCs and promotes their production of IL-6 and therefore the production of Th17 cells (Li *et al.*, 2017).

1.3.2.3. Neutrophil function

The most obvious symptoms that MST1-deficient patients suffer as well as the dramatic decrease of circulating lymphocytes can be explained by the role that MST1 plays in T cells. However, some other affectations such as recurrent acute bacterial infections and the observable transient neutropenia in some of the patients also put the focus on neutrophils. The study of neutrophil trafficking in an *in vivo* TNFα-induced peritonitis model revealed defective transmigration of *Mst1*$^{-/-}$ murine neutrophils and their incapability to penetrate the vascular basement membrane, since during extravasation *Mst1*$^{-/-}$ neutrophils they were accumulating between the endothelium and the basement membrane (Kurz, Catz and Sperandio, 2018). The efficiency of the whole neutrophil recruitment cascade is highly dependent on the correct function of the intracellular vesicle trafficking machinery, which is governed by the Rab protein family (Masgrau-Alsina, Sperandio and Rohwedder, 2020). Most neutrophil proteins that are important for the neutrophil basement membrane penetration such as VLA-3 (integrin $\alpha_3\beta_1$), VLA-6 (integrin $\alpha_3\beta_1$), and NE are contained in primary and secondary granules (Wang *et al.*, 2005; Ley *et al.*, 2007). These vesicles are the last to be secreted after neutrophil activation and Rab27a mainly controls their exocytosis. *Mst1*$^{-/-}$ mice were reported to have deficient mobilisation of Rab27a-positive vesicles in response to PECAM-1/ICAM-1/CXCL1 (Kurz *et al.*, 2016). It was shown that MST1 directly or indirectly interacts with Rab27a effector JFC1 and contributes to bring vesicles containing VLA-3, VLA-6, and NE to the cell surface (Kurz *et al.*, 2016) (Figure 9C).

1.3.2.4. ROS production in phagocytes

MST1 contributes to ROS production in many cell types. For instance, in a cardiac ischemia/reperfusion model MST1 gets upregulated in cardiomyocytes correlating with ROS production, and the genetic ablation of MST1 contributes to the reduction of the infarcted area (Yu *et al.*, 2019). In phagocytes, MST1 takes part in the juxtaposition of bacteria-containing phagosomes with the mitochondria. Phagocytes lacking MST1/2 phagocyte less bacteria and the bacteria live longer in the phagosomes (Geng *et al.*, 2015) (Figure 9D).

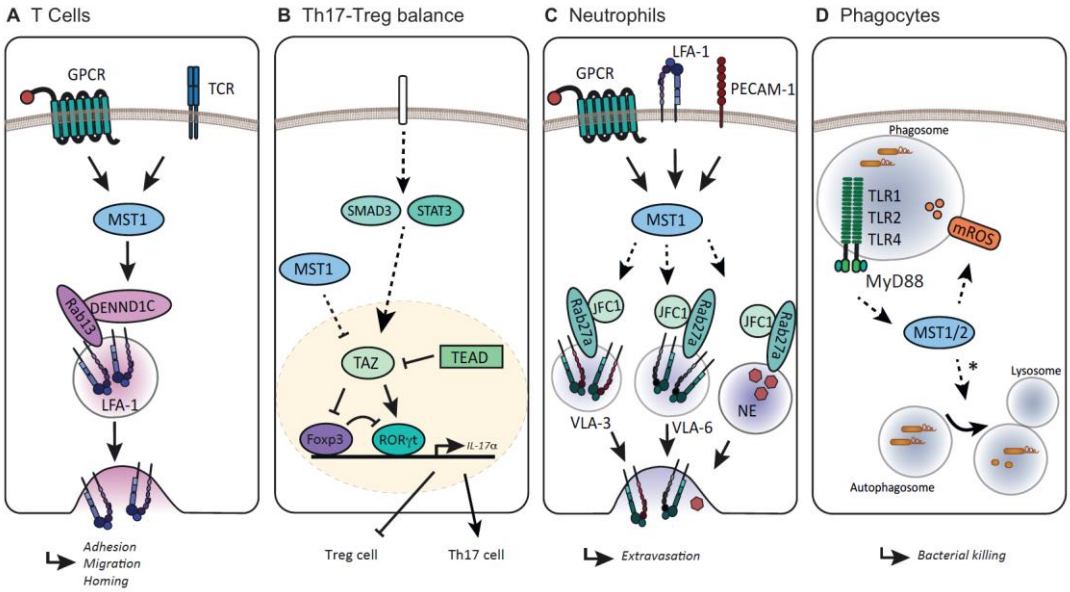

Figure 9. Non-canonical MST1 pathways. (**A**) In T cells, MST1 interacts with Rab13 effector DENND1C and contributes to the mobilization of LFA-1 containing vesicles to the cell membrane. (**B**) The absence of MST1 contributes to TAZ dependent activation of RORγt and the differentiation of naïve $CD4^+$ T cells towards pro-inflammatory Th17 cells. (**C**) In neutrophils, MST1 controls the mobilization of VLA-3, VLA-6, and NE-containing vesicles to the surface through interaction with Rab27a effector JFC1. (**D**) MST1 contributes to the juxtaposition of phagosomes to ROS-productive mitochondria. Figure taken from Kurz, Catz and Sperandio, 2018.

2. Aim of the thesis

Human MST1 deficiency causes a primary immunodeficiency disorder resulting in lymphopenia and intermittent neutropenia that starts to manifest during infancy and gravely compromises the life of those suffering it. In the past years, some investigations have contributed to the better understanding of the role that MST1 plays in immune cells but an efficient treatment to potentially cure the disease other than hematopoietic stem cell transplantation (HSCT) has not been described yet. In addition, reported cases of HSCT in MST1 deficient patients revealed a reduced success rate compared to the normal efficiency of the procedure in pediatric patients (Cagdas *et al.*, 2021). In my thesis work, I aim to further investigate the function of MST1 in neutrophils and evaluated the role MST1 plays in the G-CSFR signalling cascade, which is a key regulator of neutrophil development in the bone marrow and mobilisation to the circulation. Hypothesising that MST1 regulates neutrophil mobilisation, I used MST1 deficient mice and evaluated the *in vivo* response of neutrophils to the cytokine G-CSF and through *in vitro* approaches I intended to elucidate how MST1 modulates the G-CSFR signalling pathway on the molecular level.

3. Materials

3.1. Animal experiments

All mice used had a C57BL/6 background and were bred in the Core Facility of Animal Models in the Biomedical Center of Munich. The knockout strain *Mst1*$^{-/-}$ and the knockin strain *Lyz2*^{eGFP} (neutrophil reporter strain) were generated in the laboratory of Prof. Dr. Dae-Sik Lim (KAIST, Daejon, KR) and in the laboratory of Prof. Dr. Thomas Graf (CRG, Barcelona, ES) respectively. The *Mst1*$^{-/-}$*Lyz2*^{eGFP} strain was generated by crossbreeding. Wild type mice were provided by Charles River (Wilmington, US). All experiments were conducted under the approval of the Regierung von Oberbayern (ROB) and local authorities (project number ROB-55.2-2532.Vet_02-17-102).

3.1.1. Genotyping

All primers were provided by Metabion (Planegg, DE).

Genotype	Primer	5'-3'
Mst1	Forward	GTCCATAAGGTTCTAGCGTG
Mst1	Reverse Mst1$^{-/-}$	AGGTGTGGCACAATCGCATG
Mst1	Reverse WT	ATGCTCCAGACTGCCTTGGG
Lyz2^{GFP}	Forward	AGGCTGTTGGGAAAGGAGGG
Lyz2^{GFP}	Reverse Lyz2^{GFP}	GTCGCCGATGGGGGTGTT
Lyz2^{GFP}	Reverse WT	TCGGCCAGGCTGACTCCATA

3.1.1.1. Genotyping protocol

Mst1 and *Lyz2*	35x
Initial Denaturation	3m 95ºC
Denaturation	15s 95ºC
Annealing	15s 60ºC
Extension	15s 75ºC
Final Extension	7m 72ºC

3.1.2. mRNA isolation

Primer	Genotype	5'-3'
Mst2-A	Forward	TCCATTCGGCCCTTCTTTCT
Mst2-A	Reverse	TCAGATGCAGCCTTCTCCAA
Mst2-B	Forward	ATGAGTGAAGGAGCCCAGAC
Mst2-B	Reverse	CTCACGCATGCTCTGATCAC

3.1.2.1. cDNA obtaining protocol

Mst2	35x
Initial Denaturation	3m 95ºC
Denaturation	15s 95ºC
Annealing Primers A	15s 58ºC
Annealing Primers B	15s 60ºC
Extension	15s 72ºC
Final Extension	7m 72ºC

3.2. Cell lines

Mouse embryonic cell line 3T3 (Park *et al.*, 2012) was generously provided by AG Walzog (BMC, Planegg-Martinsried, DE) and used as a positive control for MST2 detection.

3.3. Buffers and solutions

3.3.1. Buffers for flow cytometry and fluorescence activated cell sorting

PBS-BSA 1%	For 100ml
BSA	1g
PBS	up to 100ml
	pH 7.4

HBSS	For 100ml
BSA	250mg
HEPES	250mg
Hanks solution	up to 100ml
	250mg

Sorting Buffer	100ml
FBS	2ml
HEPES 1M	2.5ml
EDTA 0.5M	0.4ml
PBS	95.1ml
	pH 7.4

Erytrocyte Lysate Buffer	100ml
NH_4Cl	8.02g
$NaHCO_3$	0.84g
EDTA	0.37g
H_2O	up to 100ml

3.3.2. Buffers for western blot

Running buffer 10x	Final concentration	For 1L
Tris	250mM	30.3g
Glycine	1.9M	144g
SDS	1%	10g
H_2O		up to 1L

Running buffer 1x	Final concentration	For 1L
Running buffer 10x	10%	100ml
H_2O		up to 1L

Blotting buffer 10x	Final concentration	For 1L
Tris	250mM	30.3g
Glycine	1.9M	144g
H_2O		up to 1L

Blotting buffer 1x	Final concentration	For 1L
Blotting buffer 10x	10%	100ml
Methanol	2.5%	25ml
H_2O		up to 1L

Tris 0.5M pH6.8	Final concentration	For 1L
Tris	0.5M	60.57g
H_2O		up to 1L
		pH 6.8

Tris 1.5M pH8.8	Final concentration	For 1L
Tris	1.5M	181.71g
H_2O		up to 1L
		pH 6.8

TBS 10x	Final concentration	For 1L
Tris	200mM	24.4g
NaCl	1.1M	80g
H_2O		up to 1L
		pH 7.4

TBST	Final concentration	For 1L
TBS 10x	10%	100ml
Tween		500ul

Modified RIPA	Final concentration	For 250ml
Tris HCl	50mM	8.3ml
NaCl 5M	150mM	7.5ml
TritonX 100	0.1%	250ul
SDS 10%	0.5%	12.5ml
EDTA 0.5M	2mM	1ml
H_2O		up to 250ml

Laemmli buffer		For 50ml
Tris HCl		15ml
SDS		5g
Glycerol		25ml
β-mercaptoethanol		10ml

Lysis buffer	For 10ml
Modified RIPA	8ml
Laemmli buffer 5x	2ml
Protease/Phosphatase Cocktail	40µl

3.3.3. Other solutions

Anaesthesia	Concentration	For 1ml
Ketamine	125mg kg^{-1}	100µl
Rompun 2% (Xylazin)	25mg kg^{-1}	100µl
NaCl		800µl

3T3 Cells medium	Final concentration	For 1L
RPMI		
FCS	10%	
Pen/Strep	1%	

Neutrophil isolation recommended medium	For 100ml
FBS	2ml
EDTA (0.5M)	0.1ml
PBS	97.9ml
	pH 7.4

3.4. Substances

3.4.1. Recombinant proteins

Substance	Company
mG-CSF	ImmunoTools
mTNFα	R&D

3.4.2. Fluorescent substances

Substance	Company
CellTracker™ Green CMFDA Dye	Invitrogen/Thermo Fisher Scientific
CellTracker™ Red CMTPX Dye	Invitrogen/Thermo Fisher Scientific
eBioscience Fixable Viability Dye eFluor™780	Invitrogen/Thermo Fisher Scientific
Qtracker™ 705 Vascular Labels	Invitrogen/Thermo Fisher Scientific
SYTOX™ Green Dead Cell Stain	Invitrogen/Thermo Fisher Scientific

3.4.3. Other substances

Substance	Company
2-Mercaptoethanol	Sigma-Aldrich
2-Propanol	Sigma-Aldrich
AccuStart™ II PCR Super Mix	Quantabio
Accutase® Cell Detachment Solution	Corning
Acrylamide	AppliChem
Agarose	Nippon Genetics Europe
Ammonium Chloride (NH_4Cl)	Merck
Ammonium Peroxodisulfate (APS)	AppliChem
Aqua	Braun
BD FACS™ Lysing Solution	BD
Bepanthen®	Bayer
Bromophenol Blue	Sigma-Aldrich

Chamaleon® Duo Pre-stained Protein Ladder	LI-COR
CountBright™ Absolute Counting Beads	Invitrogen/Thermo Fisher Scientific
Dymethil Sulfoxyde (DMSO)	Sigma-Aldrich
DNA Ladder 100pb	perQLab
Ethylenediaminetetraacetic Acid (EDTA)	Merck
Fetal Bovine Serum (FBS)	Sigma-Aldrich
Forene® (Isofluorane)	AbbVie
Gibco™ RPMI 1640 Media	Sigma-Aldrich
Glycerol	Roth
Glycin	Bernd Kraft
Hair Removal Creme	Veet
Hanks Solution	Apotheke Klinikum der Uni München
Heparin	Sigma-Aldrich
HEPES	Sigma-Aldrich
Histoacryl®	B. Braun
Hydrocloric Acid (HCl)	Millipore
Ketaset (Ketamine)	Zoetis
Methanol	AppliChem
NaCl Solution (10ml, 50ml)	Fresenius
Novalgin® (Metamizol)	Sanofi
Odyssey® Blocking Buffer (TBS)	LI-COR
Penicillin/Streptomycin Solution 100X	Corning
Percoll®	Sigma-Aldrich
Phosphate-Buffered Solution (PBS)	Apotheke Klinikum der Uni Muenchen
Protease/Phosphatase Inhibitor Cocktail 100X	Cell Signalling
Rompun 2%	Bayer
RPMI 1640 Medium	Sigma-Aldrich
Sodium Bicarbonate ($NaHCO_3$)	Merck
Sodium Deodecyl Sulfate (SDS)	Roth

Sodium Hydroxide (NaOH)	
TEMED	AppliChem
Tris(hydroxymethyl)-aminomethan	Merck Millipore
Triton® X-100	AppliChem
Türks solution	Merck
Tween® 20	Merck
UltraComp Beads™	Invitrogen

3.5. Antibodies

3.5.1. Antibodies for flow cytometry and FACS

Antibody	Conjugate	Host	Isotype	Clone	Company
Lineage cocktail	PE	Rat and Armenian Hamster	AH IgG Rat IgG2b Rat IgG2a	145-2C11 RB6-8C5 RA3-6B2 Ter-119 M1/70	BioLegend
CD3e	PE	Hamster	IgG	145-2C11	BioLegend
CD11a (LFA-1)	PerCP-Cy5.5	Rat	IgG2a k	M17/4	BioLegend
CD11b (Mac-1)	PE	Rat	IgG2b k	M1/70	BioLegend
CD11b (Mac-1)	PE-Cy7	Rat	IgG2b k	M1/70	BioLegend
CD11b (Mac-1)	AF 700	Rat	IgG2b k	M1/70	BioLegend
CD44	BV 570	Rat	IgG2b k	IM7	BioLegend
CD45	PerCP-Cy5.5	Rat	IgG2b k	30-F11	BioLegend
CD45	APC	Rat	IgG2b k	30-F11	BioLegend
CD45R (B220)	PE	Rat	IgG2a k	B220	BioLegend
CD49d (Integrin α4, VLA-4)	FITC	Rat	IgG2b k	R1-2	Biolegend
CD90.2 (Thy-1.2)	PE	Rat	IgG2b k	30-H12	BioLegend
CD114 (G-CSFR)	AF 647	Rat	IgG2a	723806	R&D
CD115 (M-CSFR)	PE	Rat	IgG2a k	AFS98	BioLegend
CD115 (M-CSFR)	APC	Rat	IgG2a k	AFS98	BioLegend
CD117 (c-Kit)	BV 421	Rat	IgG2a k	2B8	BioLegend
CD117 (c-Kit)	APC	Rat	IgG2b	2B8	eBiosciene

CD162 (PSGL-1)	PerCP-Cy5.5	Rat	IgG1	2PH1	Pharmigen
CD182 (CXCR2)	APC	Rat	IgG2a	242216	R&D
CD182 (CXCR2)	APC	Rat	IgG2a k	SA044G4	BioLegend
CD184 (CXCR4)	FITC	Rat	IgG2b	2B11	Pharmigen
Integrin β7	APC/Fire™750	Rat	IgG2a k	FIB504	BioLegend
Nk1.1	PE	Mouse	IgG2a k	PK136	BioLegend
Ly6A/E (Sca-1)	PB	Rat	IgG2a k	D7	BioLegend
Ly6A/E (Sca-1)	PE	Rat	IgG2a	D7	eBioscience
Ly6G/C (Gr-1)	PB	Rat	IgG2b	RB6-8C5	BioLegend
Ly6G/C (Gr-1)	PE-Cy7	Rat	IgG2b k	RB6-8C5	BioLegend
Ly6G	PB	Rat	IgG2a k	1A8	BioLegend
Ly6G	AF 488	Rat	IgG2a k	1A8	BioLegend

3.5.2. Isotype antibodies for flow cytometry and FACS

Antibody	Conjugate	Host	Isotype	Clone	Company
IgG1	PerCP-Cy5.5	Rat	IgG1 k	RTK2071	BioLegend
IgG2a k	PB	Rat	IgG2a k	RTK2758	BioLegend
IgG2a k	PerCP-Cy5.5	Rat	IgG2a k	RTK2758	BioLegend
IgG2a k	APC	Rat	IgG2a k	RTK2758	BioLegend
IgG2a k	AF 647	Rat	IgG2a k	RTK2758	BioLegend
IgG2a k	APC/Fire 750	Rat	IgG2a k	RTK2758	BioLegend
IgG2b	FITC	Mouse	IgG2b		Santa Cruz
IgG2b k	BV 570	Rat	IgG2b k	RTK4530	BioLegend
IgG2b k	FITC	Rat	IgG2b k	RTK4530	BioLegend
IgG2b k	APC	Rat	IgG2b k	eB149/10HS	eBiosciene

3.5.3. Primary antibodies for western blot

Antibody	Host	Clone	Company
Akt	Mouse	40D4	Cell Signalling
Phospho-Akt	Rabbit	D9E	Cell Signalling
GAPDH	Mouse	6C5	Merck Millipore
JAK1	Mouse	D1T6W	Cell Signalling
Phospho-JAK1	Rabbit	DZN4Z	Cell Signalling
JAK2	Rabbit	D2E12	Cell Signalling
Phospho-JAK2	Rabbit	C80C3	Cell Signalling
MST1	Rabbit	Polyclonal	Cell Signalling
MST2	Rabbit	Polyclonal	Cell Signalling
Phospho-MST1/MST2	Rabbit	E7U1D	Cell Signalling
p38 MAPK	Rabbit	Polyclonal	Cell Signalling
Phospho-p38 MAPK	Rabbit	Polyclonal	Cell Signalling
p44/42 MAPK (ERK1/2)	Mouse	L34F12	Cell Signalling
Phospho-p44/42 MAPK (ERK1/2)	Rabbit	D13.14.4E	Cell Signalling
STAT3	Mouse	124H6	Cell Signalling
Phospho-STAT3	Rabbit	D3A7	Cell Signalling
STAT5	Rabbit	D206Y	Cell Signalling
Phospho-STAT5	Mouse	14H2	Cell Signalling

3.5.4. Secondary antibodies for western blot

Antibody	Conjugate	Host	Company
Mouse	IRDye 680CW	Goat	LI-COR
Mouse	IRDye 800CW	Goat	LI-COR
Rabbit	IRDye 680CW	Goat	LI-COR
Rabbit	IRDye 680CW	Donkey	LI-COR
Rabbit	IRDye 800CW	Goat	LI-COR

3.6. Kits

Kit	Company
Mouse G-CSF Quantikine® ELISA Kit	R&D Systems
EasySep™ Mouse Neutrophil Enrichment Kit	STEMCELL Technologies
EasySep™ Mouse LSK Enrichment Kit	STEMCELL Technologies
eBioscience™ BrdU Flow Kit APC	BD Pharmigen
eBioscience™ Foxp3/Transcription Factor Staining Buffer Set	Invitrogen
RNeasy® Mini Kit	Qiagen

3.7. Equipment

Machine	Company
5427R Centrifuge	Eppendorf
572 Precision Balance	Kern
Biometra TAdvanced Thermocycler	Analytik Jena
CKX41 Inverted Microscope	Olympus
CytoFLEX S Flow Cytometer	Beckman Coulter
Darkbox type 4	Unit One electronics
Digital Camera C4742-80	Hamamatsu
FACSAria™ III Cell Sorter	BD
FlowSafe® Flow Hood	Brenner
Galaxy® 170 S CO_2 Incubator	New Brunswick
Gallios Flow Cytometer	Beckman Coulter
HERAfreeze™ HFU T Series Ultra-Low Temperature Freezer	Thermo Fisher Scientific
IsoTec 4 Isofluorane Vaporizer	Ohmeda
LSRFortessa™ Flow Cytometer	BD
Mastercycler® Thermocycler	Eppendorf
Megafuge™ 8 Centrifuge	Thermo Fisher Scientific
Mini-PROTEAN® Tetra Handcast System	BIO-RAD
MP220 pH Meter	Mettler Toledo
Multifuge™ X3R Centrifuge	Thermo Fisher Scientific
MultiRad 225	Precision X-Ray
NanoDrop™ 2000	Thermo Fisher Scientific
Neubauer Chamber	
Odyssey® CLx Imaging System	LI-COR
PowerPac™ Power Supply	BIO-RAD
ProCyte Dx Hematology Analyser	IDEXX
Rotina 420R Centrifuge	Hettich Zentrifugen

SBC 32 Analytical Balance	Scaltec
Spark® Microplate Reader	TECAN
TCS SP8 MP Multiphoton Microscope	Leica
Wet/Tank Blotting Systems	BIO-RAD

3.8. Consumables

Consumables	Company
Cell Culture Dishes (60, 100mm)	Corning, Falcon
Centrifuge Tubes (15, 50ml)	Corning, Falcon
Cryotube™ Vials	Thermo Fisher Scientific
Discardit™ II Syringes (2, 5, 10, 20ml)	BD
EASYstrainer™ Cell strainer 40µm	Greiner
Eppendorf Tubes® (1.5, 2, 5ml)	Eppendorf
Flasks (75cm^2)	Corning, Falcon
Immobiolion®-E PVDF Membrane	Merck Millipore
Inject®-F Fine-dose Syringes	B. Braun
Microcapillary Pipettes (15µl)	Drummond Scientific
Microlance™ Needles (several sizes)	BD
Microtainer® Blood Collection Tubes	BD
Omnican® Insulin Syringes	B. Braun
Parafilm	Bemis
Polythene Tubing 0.40mm	SIMS PORTEX
Round Bottom Tubes (5ml)	Corning, Falcon
Serological Pipettes (25ml)	Greiner Bio-One
Sterican® Needles (several sizes)	B. Braun
Stripette® Serological Pipettes (5, 10ml)	Costar
TipOne® Pippete Tips (10, 200, 1000µl)	STARLAB
Vasco® Nitril Soft Blue	B. Braun

3.9. Software

Software	Company
Excel	Microsoft
Fiji	ImageJ
FlowJo Analysis Software	BD
ImageStudio ™ Lite	LI-COR
Imaris8	Bitplane
Inkscape	Inkscape
Pixelmator	Pixelmator Team Ltd.
Prism9	GraphPad

4. Methods

4.1. Tissue isolation

4.1.1. Peripheral blood

Blood was collected via the retro-orbital venous sinus with 10µL glass capillaries into EDTA-coated or serum tubes. Prior to collection, mice were anaesthetised either with an intraperitoneal combined injection of 125mg·kg^{-1} ketamine and 12,5mg/kg^{-1} xylazin or using a vaporizer that provided 5% isoflurane in a chamber with an oxygen flow rate of 1,6L·ml^{-1}. For experiments that required to temporarily keeping the mouse alive and awake, 5mg of metamizol was orally administrated.

4.1.2. Bone marrow

Mice were sacrificed by cervical dislocation and long bones (femur, tibia, hip and humerus) were collected. Both bone ends were cut to perfuse the bone marrow with ice cold PBS using a 23G neede. The perfused solution was filtered using a 40µm cell strainer and kept at 4ºC for further processing.

4.1.3. Spleen

Whole spleen was torn into pieces, smashed, and filtered through a 40µm strainer. The cell solution was kept at 4ºC for further processing.

4.2. Cell isolation

4.2.1. Immunomagnetic cell separation

Primary mouse Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells and neutrophils were purified from mouse bone marrow using EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit and EasySep™ Mouse Neutrophil Enrichment Kit following the manufacturer instructions. Both kits allow the isolation of the desired cell type from single cell bone marrow suspensions by negative selection. Unwanted cells are targeted with biotinylated antibodies against non-LSK (CD5, CD11b, CD19, CD45R/B220, Ly6G/C (Gr-1), TER119, 7-4) and against non-neutrophils.

Streptavidin-coated magnetic particles bind to the labelled cells through the antibody and with the cells are finally separated with the use of a magnet.

4.2.2. Cell sorting

LSK cells were sorted using the BD FACS AriaIII™ when high purity was required. The gating used in this case is described in the "Flow Cytometry and Fluorescent Activated Cell Sorting (FACS)" section.

4.3. mRNA isolation

mRNA to detect *Mst2* was isolated from sorted LSK cells with the RNeasy® Mini Kit and kept at a concentration of 100mg/µl. The obtained cDNA was run through an electrophoresis gel for 1h at 110V.

4.4. Cell culture

4.3.1. 3T3 cells

Mouse embryonic line 3T3 were used as control for MST2 detection and cultured following the standard protocols of cell culture at 37ºC and 5% CO_2, changing the medium regularly and passing the cells 1:4 when the confluence was high enough.

4.5. Cell counting

4.5.1. Hematology analyzer

For obtaining blood counts during *in vivo* experiments, a sample of whole blood of each individual was analysed using the ProCyte Dx Hematology analyser (Figure 10).

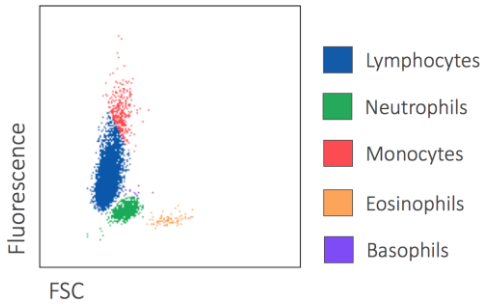

Figure 10. Representative plot of blood cell counts read by a hematology analyser. Gating strategy indicated in the legend.

4.5.2. Hemocytometer

For cell-stimulation during *in vitro* experiments, 10µl of the cell suspension was diluted into 90µl of Türks solution. 10µl of the dilution was introduced in a Neubauer counting chamber. Cells were counted using the inverted microscope and the absolute numbers extrapolated to the sample.

4.5.3. Counting beads

For flow cytometry experiments, total cell numbers were counted by introducing a known volume of CountBright™ counting beads into a known final volume of sample. The initial counting beads concentration allowed us to extrapolate the absolute number of analysed cells as described by the manufacturer.

4.6. Stimulation of isolated neutrophils with G-CSF

For the G-CSFR expression and the screening for expression and phosphorylation pattern of its downstream molecules, isolated neutrophils were resuspended in HBSS at a concentration of 10^7 cells/ml and stimulated with 1µg/ml rmG-CSF at 37ºC for 15, 30, 60, or 120 minutes. After the stimulation, cells were washed and further processed for flow cytometry or western blot.

4.7. Flow cytometry and fluorescence activated cell sorting (FACS)

All samples were incubated at 4ºC to prevent unspecific antibody binding to Fc receptors. Bone marrow and peritoneal cavity cells were incubated in either PBS-BSA 1% or HBSS. Blood cells were directly stained in whole blood with heparin and after incubation red blood cells were lysed using an ammonium-based erythrocyte lysis buffer over 10 minutes on ice.

Flow cytometry protocols were elaborated with unstained and single stained samples or UltraComp eBeads™. For multi-color panels, FMO controls were used to set the proper gatings during the analysis.

4.7.1. LSK sorting

LSK cells were isolated by FACS from whole bone marrow samples as Lineage$^-$Sca-1$^+$c-Kit$^+$ (Figure 11).

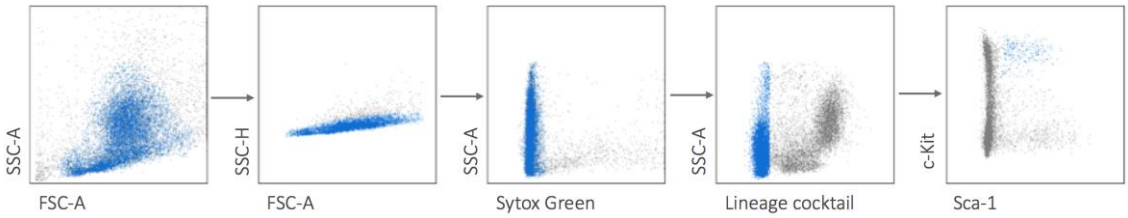

Figure 11. Representative plots of LSK identification. Blue highlight indicates the populations selected for the following step in the gating strategy.

4.7.2. Neutrophil identification

Neutrophils were identified in bone marrow and blood samples as CD45$^+$CD11b$^+$Ly6G^{high} or CD45$^+$CD11b$^+$Gr-1^{high} (Figure 12).

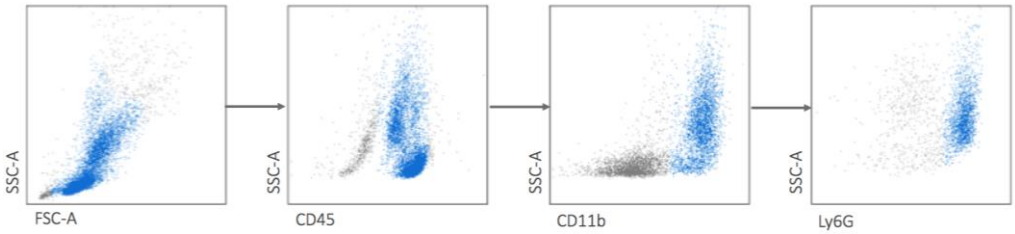

Figure 12. Representative plots of neutrophil identification. Blue highlight indicates the populations selected for the following step in the gating strategy.

Occasionally, in blood samples were I needed to identify both neutrophils and monocytes I gated neutrophils as CD45$^+$CD11b$^+$Gr-1^{high}CD115^{low}, inflammatory monocytes as CD45$^+$CD11b$^+$Gr-1^{inter}CD115^{high}, and non-inflammatory monocytes as CD45$^+$CD11b$^+$Gr-1^{low}CD115^{high} (Figure 13).

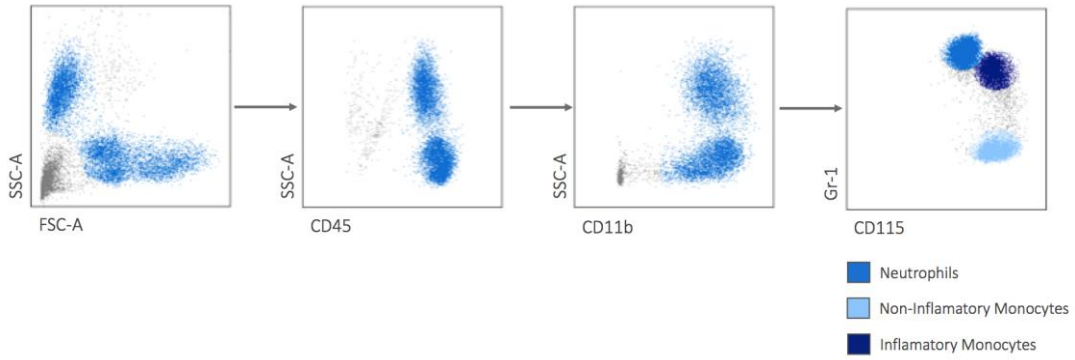

Figure 13. Representative plots of neutrophil and monocyte identification. Blue highlight indicates the populations selected for the following step in the gating strategy.

4.7.3. Neutrophil subsets in the bone marrow

Neutrophil subsets in the bone marrow were identified using the gating strategy described by Evrard et al. 2018 (Evrard *et al.*, 2018) (Figure 14).

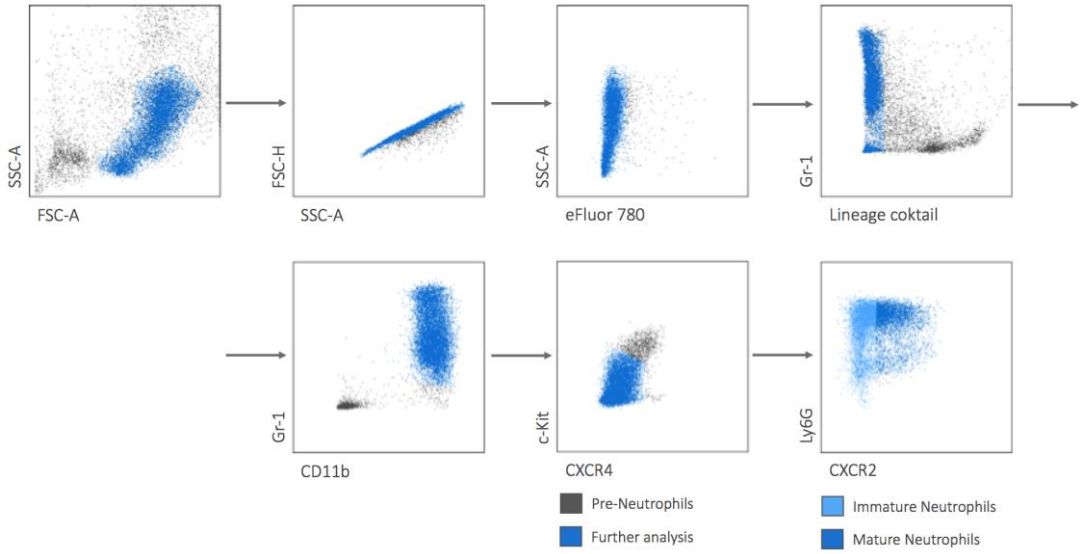

Figure 14. Representative plots of neutrophil subsets in the bone marrow panel. Blue highlight indicates the populations selected for the following step in the gating strategy. For the last plots the populations of interest are highlighted as indicated in the legend.

4.7.4. Surface protein expression

The expression of surface markers CD44, PSGL-1, integrin α_4, integrin β_7, CD11a, Ly6G, CXCR2, and CXCR4 was assessed using specific monoclonal antibodies (listed in the section 3.5.1.) and respective isotype controls (listed in the section 3.5.2.). Relative fluorescence intensity was obtained by subtracting the isotype fluorescence from the marker fluorescence.

4.7.5. Intracellular staining of STAT3

For intracellular staining of STAT3 the eBioscience™ Foxp3/ Transcription Factor Staining Buffer Set was used to permeabilize the cells following the manufacturer's indications. Prior to permeabilization cells were stained for surface markers for the proper identification of cells.

4.8. Western Blot

Bone marrow isolated neutrophils were lysed and homogenized in a lysis buffer containing Laemmli on a concentration of 10^6cells/100µL and boiled for 5 minutes to denaturize the proteins. SDS-PAGE of the lysates was performed on

10% or 12% self-casted acrylamide gels and the protein was transferred into PVDF membranes that were incubated with the corresponding antibody following the manufacturers recommended concentrations.

4.9. Intravenous injection

Intravenous injections were always performed on the lateral tail vein of mice with insulin syringes. When mice were not anaesthetized, a mouse restrainer was used to immobilize the animal and immediately released after the injection. Prior to the injection, the tail was warmed up either with warm water or the help of an infrared lamp.

4.10. Bone marrow chimeric mice

Bone marrow chimeric mice were generated to evaluate the engraftment of *Mst1*$^{-/-}$ cells and to assess the role of the non-immune compartment in the release of neutrophils from the bone marrow. Mice were X-ray irradiated twice with a dose of 5.5Gy and an interval of 24 hours between doses. The irradiator was set with the Cu 0.5 cm filter at 200kV and 20mA. Directly after the second dose, mice were transplanted with $5 \cdot 10^6$ donor cells and monitored for the following 40 days. All mice were sacrificed at day 40 post-transplantation.

4.11. Bone marrow homing assay

Intravenous injections of labelled bone marrow cells and a posterior flow cytometry analysis of labelled cells in the host bone marrow was performed to assess the homing ability of *Mst1*$^{-/-}$ cells. Bone marrow donor cells were stained either with 1:1000 CMFDA or 1:1000 CMPTX at 37ºC for 30 minutes. When only cells with the same genetic condition were injected CMFDA was used. For the co-injection of wild type and *Mst1*$^{-/-}$ cells, cells were indistinctly stained with CMFDA and CMPTX. Host mice were injected with $5 \cdot 10^6$ cells via lateral tail vein and sacrificed one hour later. Bone marrow cells were isolated and stained for surface markers before flow cytometry analysis of the samples.

4.12. Bromodeoxyuridine (BrdU) cell proliferation assay

Bone marrow and blood neutrophil proliferation in wild type and *Mst1*$^{-/-}$ mice was assessed using the APC BrdU Flow Kit following the manufacturer's

protocol. One hour after 2.5µg BrdU intravenous injection, 2.5µg rmG-CSF was administrated intravenously to promote neutrophil proliferation. Two days after injection, mice were sacrificed and blood and bone marrow samples collected. Samples were further processed for flow cytometry analysis.

4.13. TNFα induced peritonitis and G-CSF serum levels by ELISA

In order to assess the levels of serum G-CSF under normal conditions or in inflammation, wild type and *Mst1*$^{-/-}$ mice were injected with either NaCl (control) or 500ng recombinant murine TNF-α intraperitoneally to induce peritonitis. Serum was collected 4 hours after injection and peritoneal lavage performed to analyse leukocyte infiltration into the inflamed peritoneal cavity. Mouse serum was processed as indicated by the protocol of Mouse G-CSF Quantikine® ELISA and read with the Spark™ 10M microplate reader.

4.14. Neutrophil release assay

To determine the *in vivo* response of neutrophils to G-CSF, wild type and *Mst1*$^{-/-}$ mice were anesthetised with ketamine-xylazin for blood collection and intravenously injected with NaCl 0.9% (control) or 2.5µg of rmG-CSF. Four hours after injection of NaCl 0.9% or G-CSF, mice were bled again and sacrificed. Blood was further processed and absolute numbers of neutrophils were counted by flow cytometry.

4.15. Two-photon skull window imaging

Using a two-photon microscope I *in vivo* imaged the mobilization induced by G-CSF in the murine skull bone marrow. *Lyz2*^{eGFP} and *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice, as well as bone marrow chimeric mice transplanted with cells from the contrary genetic condition, were first stimulated with an intravenous injection of 2.5µg of rmG-CSF to induce neutrophil mobilisation from the bone marrow into the blood circulation. Shortly before imaging, mice were anaesthetised with ketamine-xylazin and the skull bone exposed by removing the hair and the skin over it, as well as the pericranium with the gentle help of a scalpel. A metal ring was glued on the skull bone and firmly fixed to the imaging stage in order to prevent any breathing movement. To visualize the bone marrow microvasculature, 10µL of QTracker™ 705 was administrated intravenously. An intraperitoneal catheter was

placed to inject 100mL of anaesthetics hourly to keep the mouse under anesthesia. Imaging was carried out with the TCS SP8 MP Multiphoton Microscope using the objective HC IRAPO L (25x, 1.00 W) and started two hours after rmG-CSF injection. For each mouse, an area of analysis of 70x170µm was defined surrounding a vessel and imaging acquired in 512x512 pixels with a step size of 4µm for intervals of 15s from hour 2 to hour 4 post G-CSF injection. Analysis was performed using Imaris 8 Cell Imaging Software.

4.16. Statistics

All statistical analysis and graphs were generated with GraphPad 9 software and different tests were used depending on the number of groups to compare. For comparing two independent groups an unpaired student's t-test was used whereas for the statistical analysis between more than two groups an analysis of variance (ANOVA) was performed. Depending on the number of independent variables I either conducted a one-way ANOVA with a Tukey's multiple comparison test or a two-way ANOVA with a Sidak's multiple comparisons test. Statistical significance was considered when the p-value was <0.05.

5. Results

5.1. Characterization of *Mst1*$^{-/-}$ mice

5.1.1. *Mst1*$^{-/-}$ mice have higher numbers of neutrophils in the bone marrow but not in peripheral blood or spleen

Reports on patients with loss of function mutations on *Mst1* gene highlight the presence of peripheral lymphopenia (Abdollahpour *et al.*, 2012; Nehme *et al.*, 2012) and transient neutropenia (Abdollahpour *et al.*, 2012). In *Mst1*$^{-/-}$ mice, the absence of MST1 also translates to abnormal whole blood cell counts (Figure 15A-E) due to decreased numbers of circulating lymphocytes (Figure 15B). Interestingly, I also found increased levels of peripheral eosinophils (Figure 15D), normally a sign of allergic disease or worm infection (Diny, Rose and Čiháková, 2017).

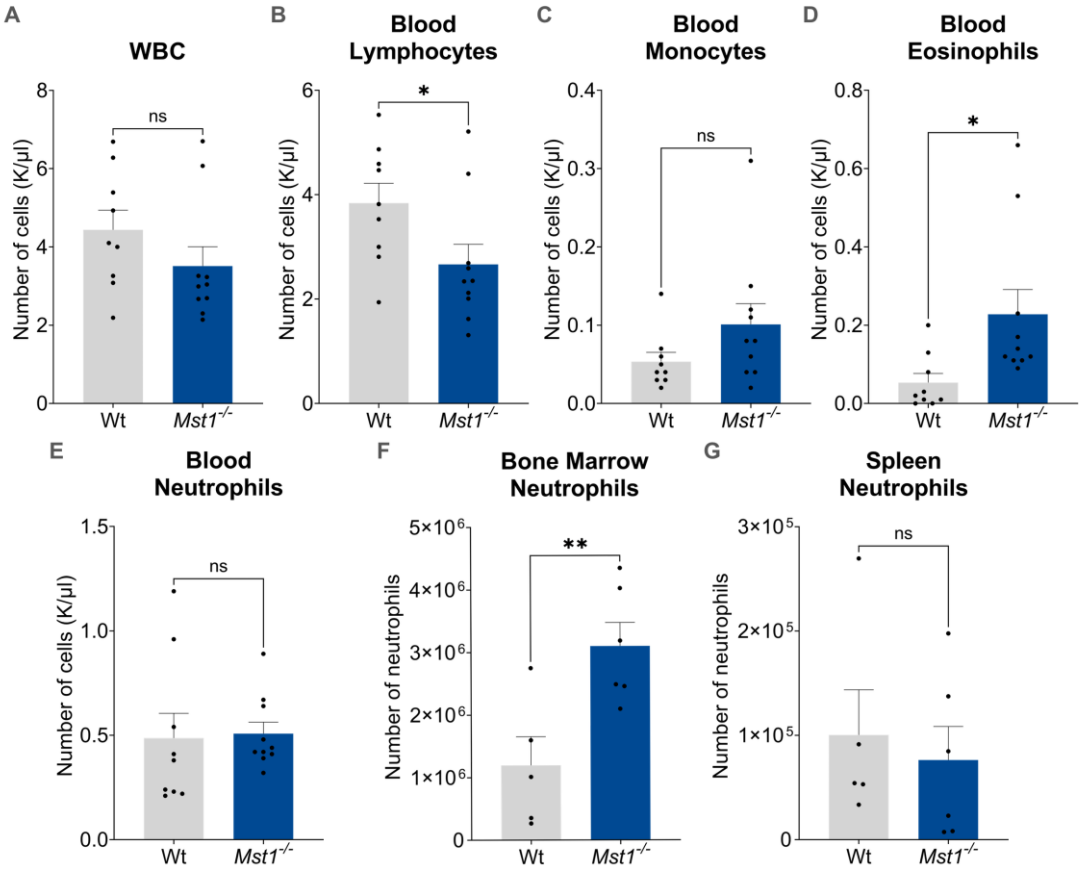

Figure 15. Whole blood cell counts and neutrophil bone marrow and spleen counts from wild type and *Mst1*$^{-/-}$ mice. (**A-E**) Blood cell counts of wild type and *Mst1*$^{-/-}$ mice obtained with the hematology analyser. Unpaired t-test; *p<0.05; n≥9. (**F-G**) Neutrophil counts in bone marrow (F) and spleen (G) obtained by flow cytometry. Unpaired t-test; **p<0.01; n≥5. *WBC – White blood cells.*

Regarding neutrophils, the analysis did not reveal an alteration on circulating neutrophil numbers (Figure 15E). Neutrophils enter the blood circulation once they have developed from granulocyte progenitors in the bone marrow and in case they do not get recruited into inflamed tissue, they get retained in clearance organs such as the bone marrow, the spleen, and the liver to get destroyed and phagocytosed. Looking at the bone marrow neutrophil numbers I found a significant increase in *Mst1*$^{-/-}$ mice (Figure 15F). However, neutrophil numbers in the spleen were unaltered (Figure 15G).

Such high numbers of neutrophils in the bone marrow that do not translate to abnormal circulating neutrophils suggest: (1) a defect in *Mst1*$^{-/-}$ neutrophils that does not allow them to fully mature and leave into the circulation, (2) an enhanced homing capacity of *Mst1*$^{-/-}$ neutrophils that retains them in the bone marrow, and/or (3) a defect in the release of bone marrow mature neutrophils into peripheral blood.

5.1.2. MST1 is expressed in LSK cells and MST2 can only be found in LSK cells and neutrophils on the mRNA level

The hypothetical role of MST1 in neutrophil development made me focusing on both LSK cells and mature neutrophils. MST1 is known to be ubiquitously expressed but even though MST1 was reported to be expressed at a protein level in neutrophils (Kurz *et al.*, 2016) there was no direct data on MST1 expression in LSK cells. Therefore I assessed the levels of the protein by western blot in wild type isolated LSK cells and confirmed the presence of MST1 (Figure 16A).

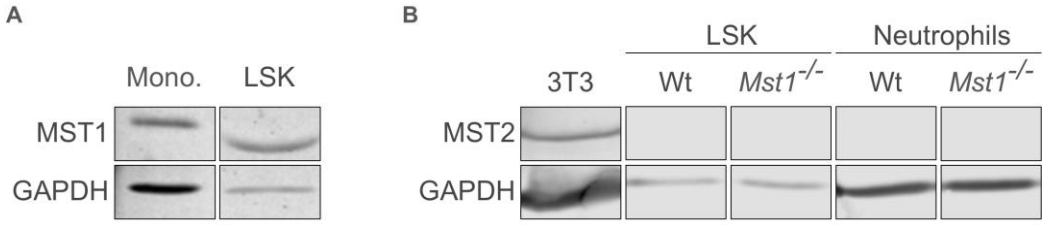

Figure 16. MST1 and MST2 expression in LSK cells and bone marrow neutrophils. (**A**) MST1 western blot performed on 30.000 sorted LSK cells and using monocyte lysates as a positive control and GAPDH as loading control. (**B**) MST2 western blot performed on 30.000 sorted LSK cells and $5{\cdot}10^5$ neutrophils using 3T3 cell line lysates as a positive control and GAPDH as loading control.

Mst1$^{-/-}$ single knockout mice still have the paralog gene *Mst2* functional. Even though MST2 is only weakly expressed, the absence of a protein can lead to the upregulation of its paralog as a way of genetic compensation (El-Brolosy and Stainier, 2017). By looking at MST2 protein expression by western blot on LSK cells and neutrophils, I was not able to detect the protein in neither wild type nor *Mst1*$^{-/-}$ cells (Figure 16B) and therefore I discarded a possible MST2 upregulation that could rescue the MST1 absence phenotype in the analysed cell types.

On the mRNA level, I could qualitatively detect Mst2 in LSK and neutrophils of wild type and *Mst1*$^{-/-}$ mice (Figure 17). At any rate, mRNA expression does not always translate to protein expression (Koussounadis *et al.*, 2015).

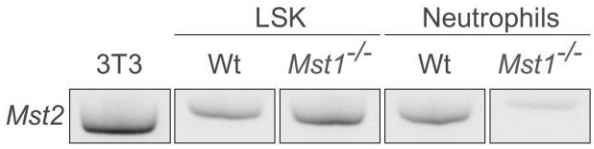

Figure 17. *Mst2* mRNA expression in LSK cells and bone marrow neutrophils. Qualitative results from *Mst2* mRNA detection on LSK cells and bone marrow neutrophils isolated from wild type and *Mst1*$^{-/-}$ mice.

5.1.3. Marker expression in LSK cells and bone marrow neutrophils

The interaction between bone marrow residing cells with vascular and stromal cells through surface proteins determine the fate of the cell. For instance, LSK cell interaction with the bone marrow niche can determine the self-renewal or the differentiation of the cell. In case of mature neutrophils, changes in surface protein expression are important to release them to the circulation or to retain them. I analysed the expression of CD44, CXCR4, PSGL-1, integrin α_4, (subunit of VLA-4), integrin β_7 (subunit of LPAM-1), and CD11a (subunit of LFA-1) by flow cytometry for both LSK cells and neutrophils (Figure 18). I didn't find any significant up- or downregulation on those molecules in LSK cells but I observed a slight increase of PSGL-1 (Figure 18F) and integrin β_7 in *Mst1*$^{-/-}$ neutrophils (Figure 18J). Since PSGL-1 is an important glycoprotein for neutrophil rolling and signalling along the recruitment cascade and integrin β_7 plays a role in cell adhesion these results pointed towards a possibly enhanced re-homing capacity of *Mst1*$^{-/-}$ compared to wild type neutrophils.

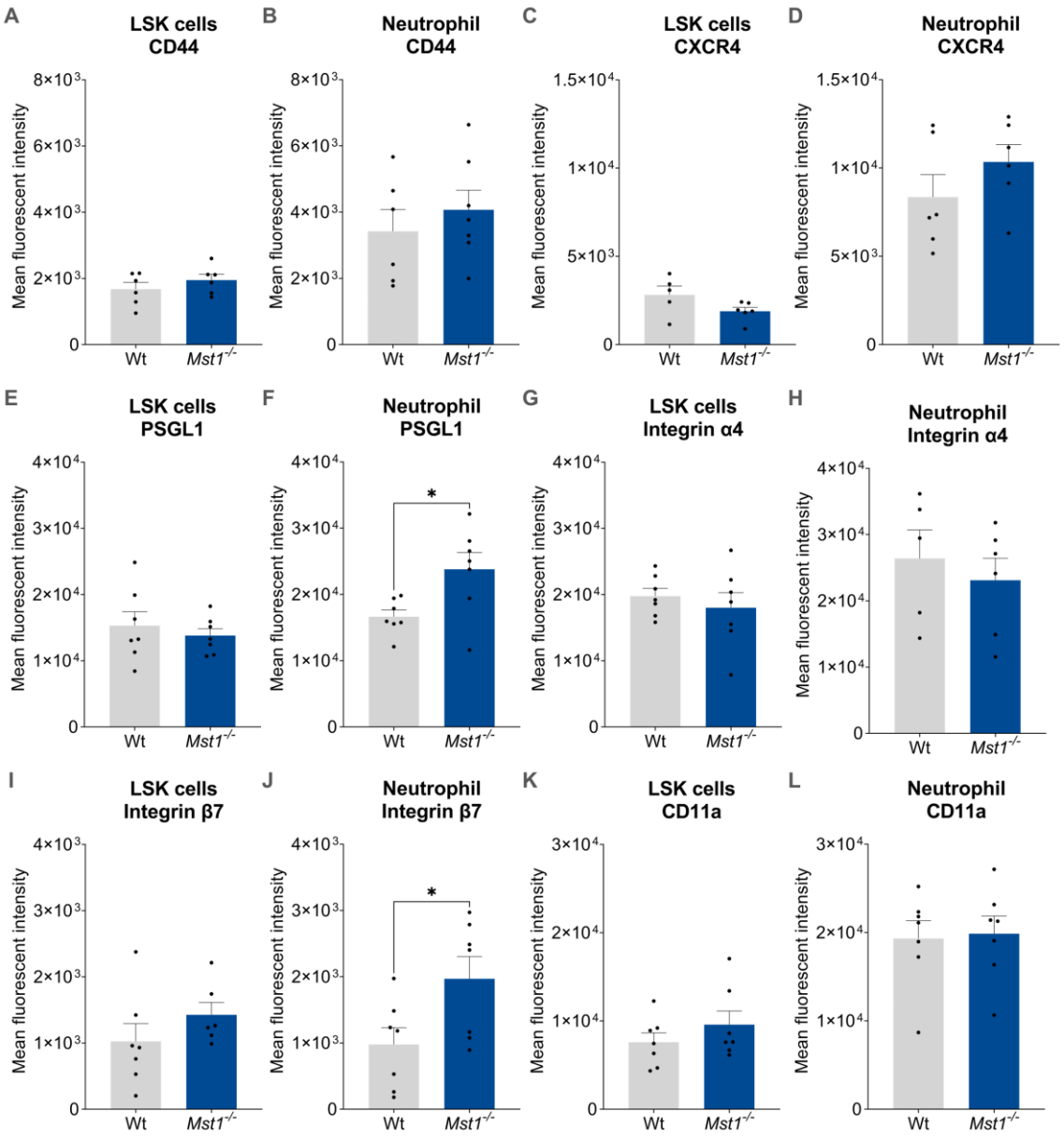

Figure 18. Expression of cell surface markers CD44, CXCR4, PSGL1, integrin α_4, integrin β_7, and CD11a in murine LSK cells and bone marrow neutrophils. (A-L) Flow cytometry analysis of cell surface markers relative to isotype controls. Unpaired t-test; *p<0.05; n≥5.

5.2. Development of *Mst1*$^{-/-}$ neutrophils

5.2.1. Bone marrow *Mst1*$^{-/-}$ neutrophils show no maturation defect but circulating *Mst1*$^{-/-}$ neutrophils express less Ly6G

A possible defect in the differentiation of myeloid precursors to mature neutrophils impairing the ability to leave the bone marrow niches and the tendency to accumulate in the niche could be an explanation for the high numbers of neutrophils in the bone marrow. To elucidate if this was the case, I analysed the ratios of the different neutrophil subsets within the bone marrow as suggested by Evrard et al. 2018 (Evrard *et al.*, 2018) (Figure 19A). In both wild type and *Mst1*$^{-/-}$ mice I found that the relative amounts of pre-neutrophils, immature neutrophils and mature neutrophils were the same (Figure 19B), suggesting that there was no maturation defect in *Mst1*$^{-/-}$ bone marrow neutrophils.

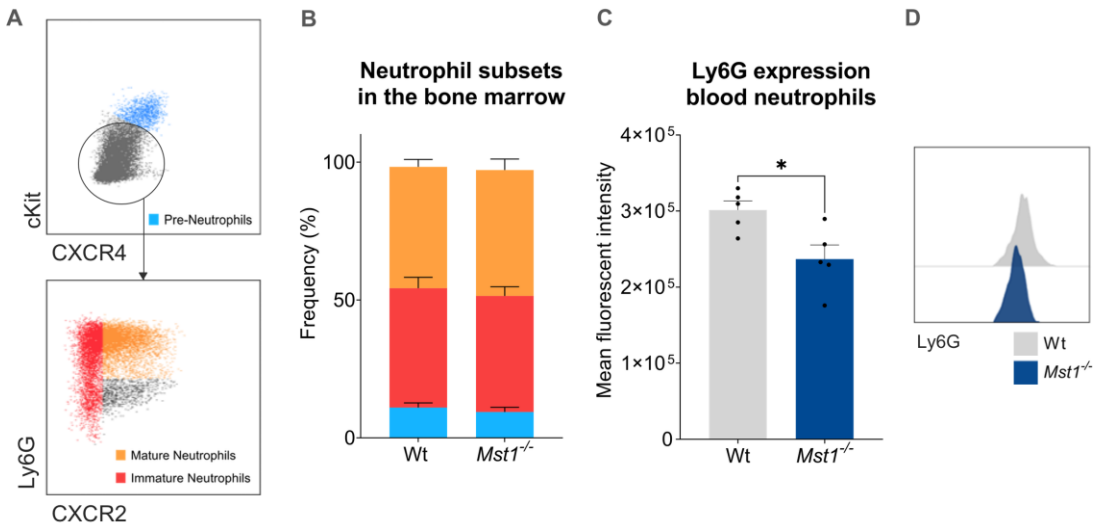

Figure 19. Neutrophil subsets in the bone marrow and maturity of circulating neutrophils. (**A**) Gating strategy to define neutrophil subsets in the bone marrow: pre-neutrophils (blue), immature neutrophils (red) and mature neutrophils (orange). (**B**) Stacked representation of the different subsets of neutrophils in the bone marrow of wild type and *Mst1*$^{-/-}$ mice. (**C**) Ly6G expression on wild type and *Mst1*$^{-/-}$ neutrophils. Unpaired t-test; *p<0.05; n=5. (**D**) Representative histogram plot of Ly6G expression on wild type and *Mst1*$^{-/-}$ neutrophils.

One of the hallmarks of neutrophil maturation is the expression of Ly6G on their surface. Freshly released bone marrow neutrophils express less Ly6G than long term circulating neutrophils (Boivin *et al.*, 2020) and therefore Ly6G expression can be used to assess the level of maturation of circulating neutrophils. Here, I found that *Mst1*$^{-/-}$ circulating neutrophils had less Ly6G expression than

wild type controls (Figure 19C-D) suggesting that *Mst1*$^{-/-}$ circulating neutrophils are less mature.

5.2.2. Bone marrow *Mst1*$^{-/-}$ neutrophils are more proliferative

The absence of MST1 in bone marrow neutrophils could be enhancing neutrophil proliferation since MST1 is known to be a suppressor of cell proliferation (Halder and Johnson, 2011). In order to assess the rates of neutrophil proliferation within the bone marrow, an injection of fluorescent labelled bromodeoxyuridine (BrdU) followed by an injection of G-CSF one hour later to stimulate cell proliferation was performed. 48 hours later I collected bone marrow and blood samples to analyse the ratio of neutrophils that incorporated the thymidine analogue (Figure 20A). In bone marrow, I observed that a significantly higher number of neutrophils incorporated BrdU (Figure 20B). The same tendency was observed in peripheral blood neutrophils (Figure 20C).

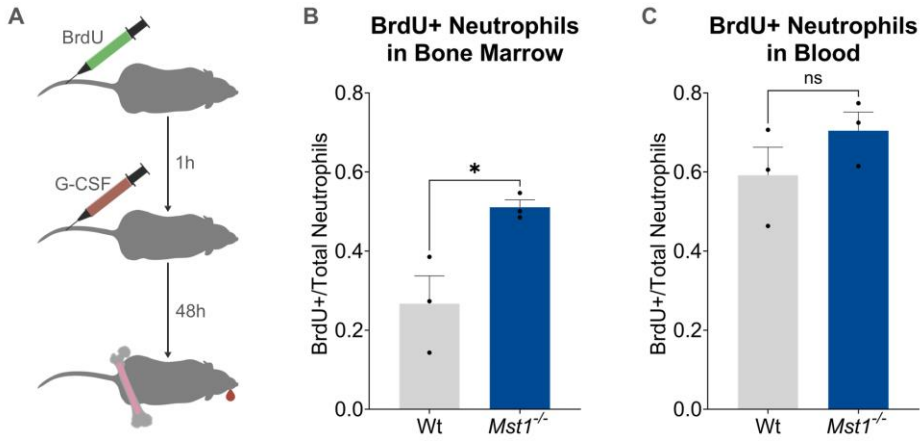

Figure 20. Neutrophil proliferation assay. (**A**) Schematic representation of the assay. (**B-C**) Ratio of BrdU+ over total number of neutrophils in bone marrow (B) and blood (C) in wild type and *Mst1*$^{-/-}$ mice. Unpaired t-test; *p<0.05; n=3.

5.3. Homing capacities of *Mst1*$^{-/-}$ hematopoietic cells

5.3.1. Wild type and *Mst1*$^{-/-}$ cells are able to repopulate an irradiated bone marrow

Next, the ability of *Mst1*$^{-/-}$ bone marrow cells to engraft the bone marrow of an irradiated host was evaluated. For this wild type and *Mst1*$^{-/-}$ donor cells were transplanted into wild type and *Mst1*$^{-/-}$ recipients and the survival of the individuals during 40 days was analysed (Figure 21A).

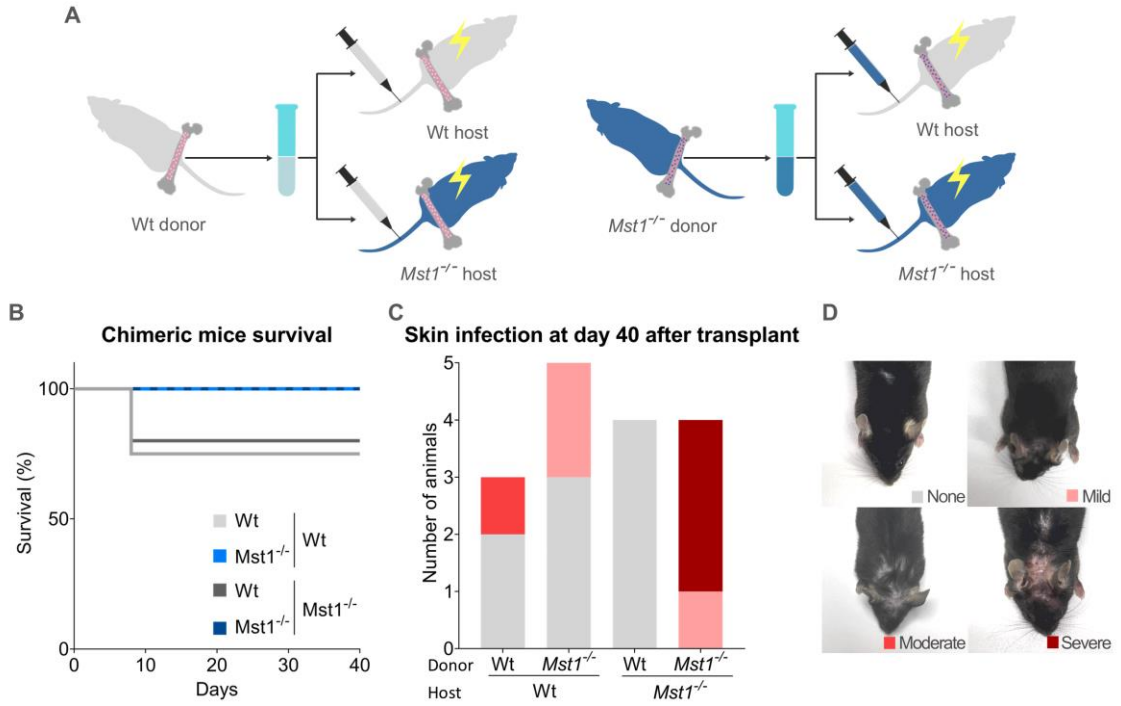

Figure 21. Bone marrow chimeric mice survival and blood cell counts. (**A**) Schematic representation of the bone marrow chimeric mice generation. (**B**) Survival rate of chimeric mice. (**C**) Presence of fungal skin infection in chimeric mice after 40 days post-transplantation, and (**D**) clinical categorization of the fungal infection by severity.

As seen in the survival curve of the bone marrow transplanted mice (Figure 21B) almost all mice in the experimental setup had a successful engraftment and survived until day 40 post-transplantation and none of the perished mice received donor *Mst1*$^{-/-}$ cells. Of note, starting from week 5 after transplantation some mice developed a dorsal skin infection that on the termination of the experiment on day 40 post-transplantation was categorized as none, mild, moderate, or severe (Figure 21D) depending on its extension. From all the experimental groups, *Mst1*$^{-/-}$ host mice that received *Mst1*$^{-/-}$ donor cells showed the highest rates of severe infection (Figure 21C) thus suggesting that even though *Mst1*$^{-/-}$ cells can engraft the bone marrow immune responses are weaker and MST1 in non-hematopoietic cells of the host may also play a role.

5.3.2. Donor *Mst1*$^{-/-}$ cells home to the host bone marrow in similar rates as wild type cells

Next, I analysed the capacity of wild type and *Mst1*$^{-/-}$ donor cells to home the bone marrow of host wild type or *Mst1*$^{-/-}$ mice (Figure 22A). By staining the

donor's isolated bone marrow cells with CellTracker™ Green CMFDA Dye or CellTracker™ Red CMTPX Dye I was able to detect the amount of wild type and *Mst1*$^{-/-}$ cells that homed into the host's bone marrow one hour after the intravenous injection. The amount of wild type and *Mst1*$^{-/-}$ donor cells detected in the host's bone marrow did not differ (Figure 22B).

To exclude any interference of wild type bone marrow cells with the homing capacity of *Mst1*$^{-/-}$ cells (Rohwedder *et al.*, 2019) I also injected wild type and *Mst1*$^{-/-}$ bone marrow cells separately into wild type recipients (Figure 22C). The flow cytometry analysis showed once more that similar amount of wild type and *Mst1*$^{-/-}$ cells homed into the host bone marrow (Figure 22D) suggesting that bone marrow homing capacity of *Mst1*$^{-/-}$ cells is functional.

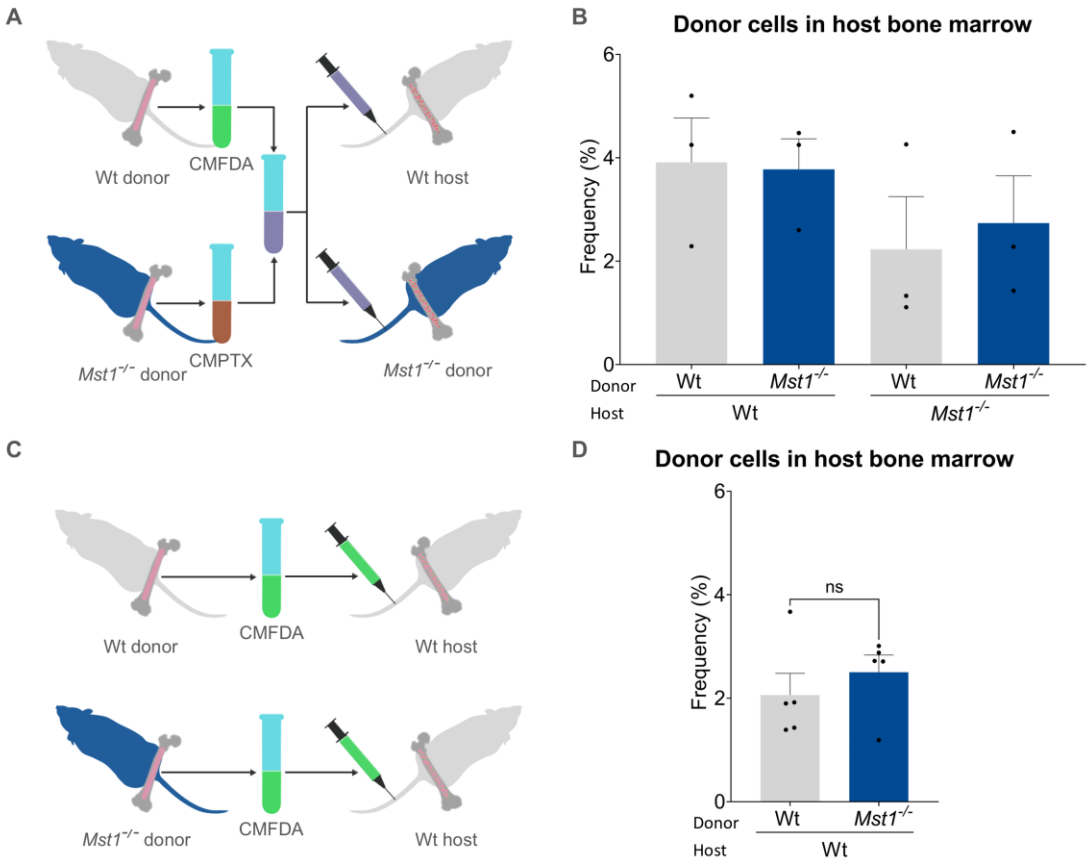

Figure 22. *In vivo* homing assay. (**A**) Wild type and *Mst1*$^{-/-}$ cells were isolated, stained with the cell tracker CMFDA or CMPTX, and injected together into a wild type or a *Mst1*$^{-/-}$ host. (**B**) Percentage of wild type and *Mst1*$^{-/-}$ donor cells that were found in the host's bone marrow. Unpaired t-test; n=3. (**C**) Wild type and *Mst1*$^{-/-}$ cells were isolated, stained with the cell tracker CMFDA, and injected separately into a wild type host. Host bone marrow was harvested and analysed one hour after the injection. (**D**) Percentage of wild type and *Mst1*$^{-/-}$ donor cells that were found in the host's bone marrow. Unpaired t-test; n=5.

5.4. Release of *Mst1*$^{-/-}$ neutrophils

5.4.1. *Mst1*$^{-/-}$ mice show increased serum levels of G-CSF both under normal conditions and under inflammation

The observations so far did not reveal any defect in *Mst1*$^{-/-}$ neutrophil development or in its ability to home to the bone marrow. The third hypothesis was that *Mst1*$^{-/-}$ neutrophils could show a defect on being released into the circulation from the bone marrow. Therefore the focus was put on G-CSF, which has a very important role in neutrophil development but is also the main cytokine regulating the release of mature neutrophils into the circulation. First, the intrinsic levels of G-CSF in murine serum under normal conditions and in inflammation were assessed. For this, I injected either NaCl 0.9% or TNFα into the mouse peritoneum to induce an inflammation. 4 hours later, blood was collected to isolate serum and run a G-CSF ELISA (Figure 23A). In order to confirm that the TNFα injection caused peritonitis I performed a peritoneal lavage to analyse by flow cytometry the numbers of infiltrated neutrophils. I observed that neutrophil infiltration was very low in NaCl 0.9% injected mice and increased in wild type mice that were injected with TNFα. In contrast, in TNFα-injected *Mst1*$^{-/-}$ mice the number of infiltrated neutrophils was lower than in wild type mice (data not shown), as reported earlier (Kurz *et al.*, 2016).

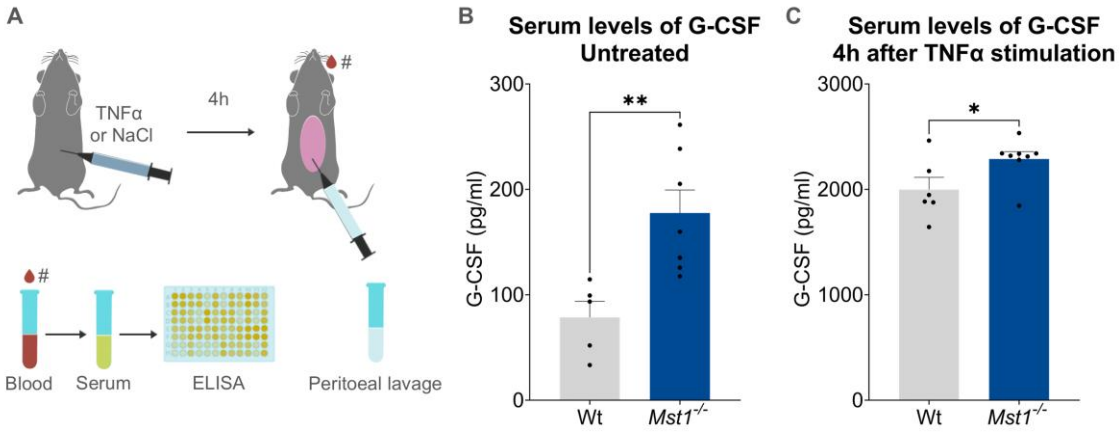

Figure 23. Serum levels of G-CSF in TNFα peritonitis. (**A**) Schematic representation of the TNFα peritonitis induction and the collection of peritoneal lavage and serum. (**B-C**) Levels of G-CSF in serum of untreated mice (B) and TNFα peritonitis-induced mice (C). Unpaired t-test; *p<0.05, **p<0.01; n≥5.

The G-CSF ELISA results revealed that G-CSF levels were increased in *Mst1*$^{-/-}$ mice both under normal conditions (Figure 23C) and during TNFα-induced inflammation (Figure 23D).

5.4.2. Neutrophil G-CSF-dependent release is impaired in *Mst1*$^{-/-}$ mice

To further evaluate the response of *Mst1*$^{-/-}$ mice to the *in vivo* stimulation of G-CSF, I collected peripheral blood before and after the intravenous injection of the cytokine or NaCl 0.9% (control) and analysed by flow cytometry the absolute numbers of circulating neutrophils (Figure 24A). Before the injection, all mice had similar numbers of circulating neutrophils (Figure 24B). After the injection, wild type mice that received G-CSF showed a significant increase of circulating neutrophils compared to wild type mice that received NaCl (Figure 24C). In contrast, G-CSF stimulation did not lead to a significant increase in neutrophil numbers in *Mst1*$^{-/-}$ mice (Figure 24C). The failure in neutrophil release in response to G-CSF suggests that *Mst1*$^{-/-}$ neutrophils have a defective response to the cytokine and do not leave the bone marrow.

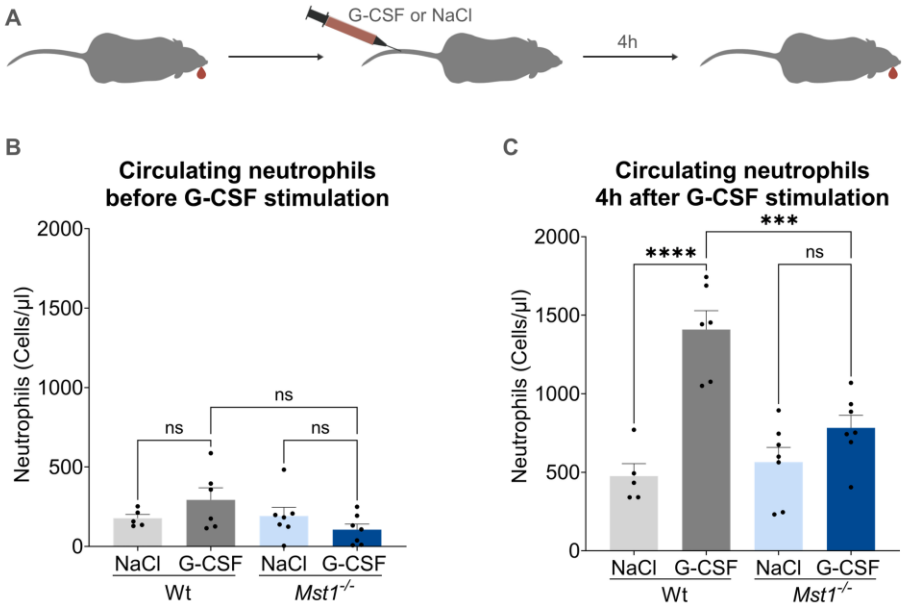

Figure 24. Neutrophil release assay. (**A**) Schematic representation of the assay. (**B-C**) Peripheral blood neutrophil counts before (B) and after (C) rmG-CSF injection. Two-way ANOVA; ****p<0.0001, ***p<0.001; n≥5.

5.4.3. G-CSF *in vivo* stimulation leads to reduced neutrophil mobilisation in *Mst1*$^{-/-}$ mice

By directly visualizing bone marrow neutrophils after *in vivo* stiumulation with G-CSF using multiphoton microscopy I aimed to uncover a mobilisation defect of *Mst1*$^{-/-}$ neutrophils from the bone marrow niche to the circulation. Accordingly I injected *Lyz2*^{eGFP} and *Mst1*$^{-/-}$*Lyz2*^{eGFP} neutrophil reporter mice with G-CSF and exposed the skull to start imaging of the skull bone marrow 2 hours after the stimulation with G-CSF (Figure 25A). In order to also evaluate MST1 function of the bone marrow stromal environment, I generated chimeric mice transplanting *Lyz2*^{eGFP} donor cells into *Mst1*$^{-/-}$*Lyz2*^{eGFP} host mice and *Mst1*$^{-/-}$*Lyz2*^{eGFP} donor cells into *Lyz2*^{eGFP} mice.

During imaging, I noticed relevant structural differences between *Lyz2*^{eGFP} and *Mst1*$^{-/-}$*Lyz2*^{eGFP} bone marrow. Vessel number and ramification were more prominent in *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice (Figure 25B) and the abundance of bone marrow niches, identified as fluorescent clusters in the bone marrow, was also higher in *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice (data not shown). To overcome these morphological differences, the imaging was carried out around a single vessel and migrating and intravasating cells were put relative to the total amount of neutrophils counted in the area of analysis. The analysis revealed a significant decreased migration of *Mst1*$^{-/-}$*Lyz2*^{eGFP} neutrophils compared to *Lyz2*^{eGFP} controls (Figure 25C) as well as a significant reduction on the number of *Mst1*$^{-/-}$*Lyz2*^{eGFP} neutrophils that intravasated and entered the circulation (Figure 25D). Neutrophil migration in chimeric-mice was not significantly different to wild type mice but a decreased migration tendency was observable in *Lyz2*^{eGFP} host mice that received *Mst1*$^{-/-}$*Lyz2*^{eGFP} cell transplant (Figure 25C), supporting the intrinsic migration defect of *Mst1*$^{-/-}$*Lyz2*^{eGFP} observable in non-chimeric mice. Interestingly, *Lyz2*^{eGFP} donor cells in a host *Mst1*$^{-/-}$*Lyz2*^{eGFP} environment did not show the intravasating numbers that did in non-chimeric *Lyz2*^{eGFP} mice, suggesting a role of MST1 in the non-hematopoietic compartment of the bone marrow vessel (Figure 25D).

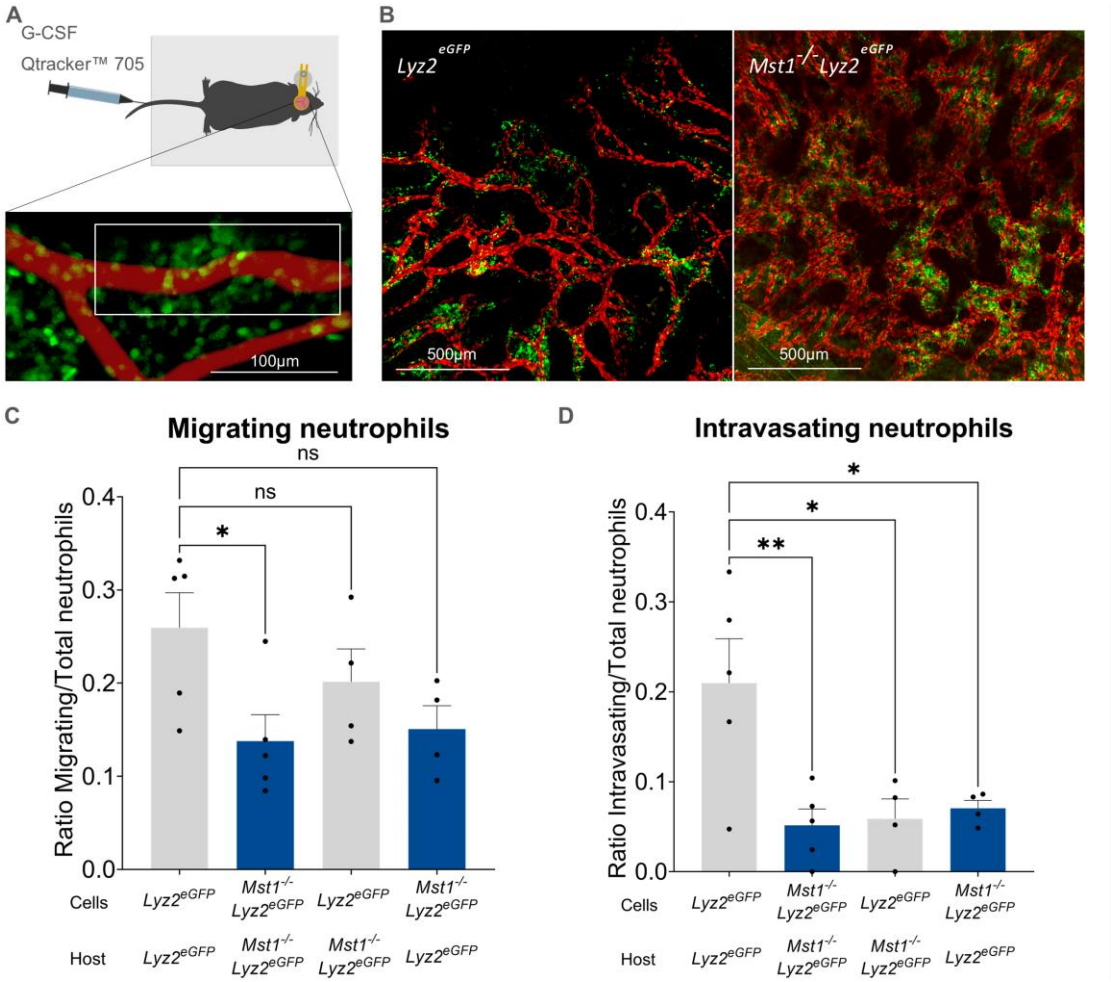

Figure 25. Multiphoton imaging of the skull bone marrow after G-CSF stimulation. (**A**) Schematic representation of the assay. Images were obtained using a reporter mouse strain *Lyz2*^{eGFP} with eGFP labelled neutrophils and labelling the vessels with Qtracker™ 705. The area of analysis surrounding the vessel is 70x170µm. (**B**) Structure of the vessel and neutrophil niche distribution of wild type and *Mst1*$^{-/-}$ mice bone marrow. (**C**) Ratio of migrating neutrophils over the total number of neutrophils at time 0 in the area of analysis of non-chimeric and chimeric mice. One-way ANOVA; *p<0.05; n≤5. (**D**) Ratio of intravasating neutrophils over the total number of neutrophils at time 0 in the area of analysis of non-chimeric and chimeric mice. One-way ANOVA; *p<0.05, **p<0.01; n≤4.

5.5. G-CSFR signalling in *Mst1*$^{-/-}$ neutrophils

5.5.1. G-CSFR expression is not altered in *Mst1*$^{-/-}$ mice

Defective G-CSF responses could be due to differential basal expression of G-CSFR on *Mst1*$^{-/-}$ neutrophils or altered signalling of the receptor. After binding the cytokine, the G-CSFR gets internalized and it is either degraded via the lysosomal pathway or shuttled back to the membrane (Dwivedi and Greis, 2017). By western blotting I analysed the expression of total G-CSFR in isolated

unstimulated and G-CSF stimulated bone marrow neutrophils (Figure 26A) and found no differential expression of the receptor (Figure 26B). Therefore, I discarded a possible alteration of G-CSFR degradation pathway in *Mst1*$^{-/-}$ neutrophils. To analyse the G-CSF receptor expression only on the neutrophil's membrane conventional flow cytometry was used. The levels of plasma membrane expression of G-CSFR did not differ in any of the timepoints of stimulation between wild type and *Mst1*$^{-/-}$ neutrophils (Figure 26C) so a possible alteration on the receptor's recycling pathway was not contemplated.

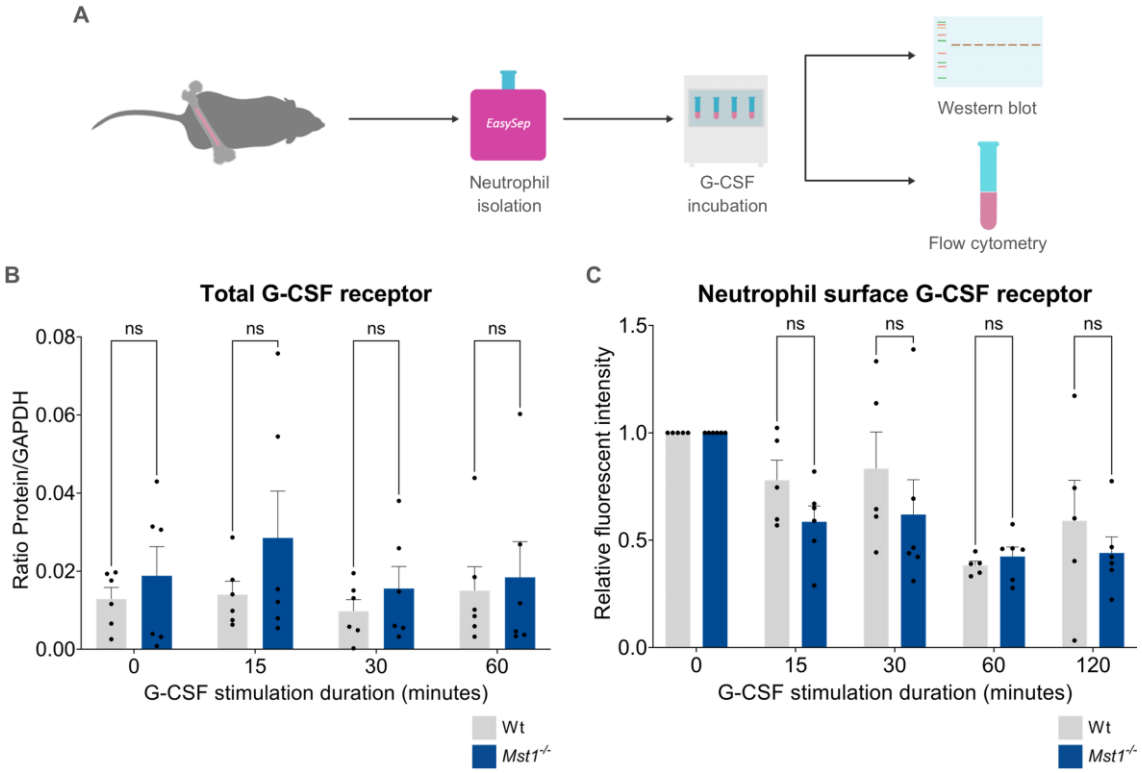

Figure 26. Neutrophil G-CSFR total and surface expression. (**A**) Schematic representation of bone marrow neutrophil isolation and stimulation. (**B**) Total amount of G-CSFR protein detected by western blot in unstimulated and 15, 30, or 60 minutes G-CSF-stimulated neutrophils. One-way ANOVA; n=6. (**C**) Relative expression of G-CSFR on the neutrophil surface analysed by flow cytometry in unstimulated and 15, 30, 60, or 120 minutes G-CSF-stimulated neutrophils. One-way ANOVA; n=5.

5.5.2. STAT3 phosphorylation after G-CSF stimulation is diminished in *Mst1*$^{-/-}$ neutrophils

The interaction of the G-CSFR with G-CSF leads to the activation of several signalling pathways, including JAK-STAT (Tian *et al.*, 1996), ERK/MAPK (Theyab *et al.*, 2021), and PI3K/Akt (Polak and Buitenhuis, 2012) pathways.

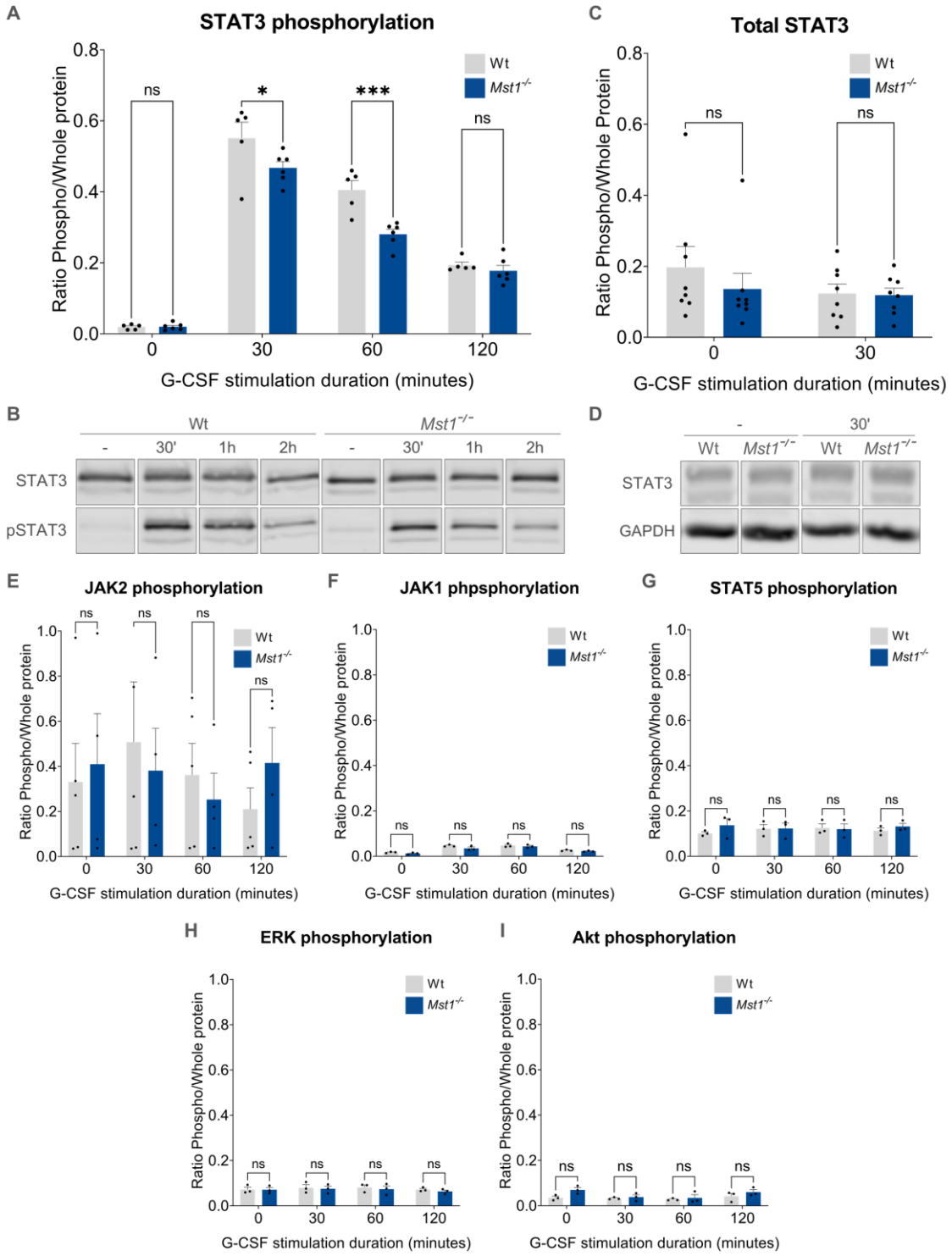

Figure 27. Phosphorylation of G-CSFR downstream signalling molecules following stimulation of bone marrow neutrophils with G-CSF. (**A**) STAT3 phosphorylation rate analysed by western blot in unstimulated and 15, 30, 60, and 120 minutes G-CSF-stimulated neutrophils. One-way ANOVA; *p<0.05, ***p<0.001; n≤6. (**B**) Representative blots of STAT3 phosphorylation analysis. (**C**) Total expression of STAT3 relative to GAPDH in unstimulated and 30 minutes stimulated neutrophils. One-way ANOVA; n=8. (**D**) Representative blots of total STAT3 levels analysis. (**E-I**) Phosphorylation rates of JAK2 (E), JAK1 (F), STAT5 (G), ERK (H), and Akt (I) in unstimulated and 15, 30, 60 and 120 minutes G-CSF-stimulated neutrophils. One-way ANOVA; n≤5.

The main proteins of these signalling pathways are activated by phosphorylation so I screened for their phosphorylation pattern in *in vitro* G-CSF-stimulated bone marrow neutrophils (Figure 27). The most relevant finding was the diminished phosphorylation and therefore activation of STAT3 in *Mst1*$^{-/-}$ neutrophils 30 and 60 minutes after exposure to G-CSF (Figure 27A-B). No differential phosphorylation pattern was observed in any other screened protein (Figure 27E-I). The decrease of phosphorylated STAT3 could also be due to less differences in *Mst1*$^{-/-}$ neutrophils total STAT3. Hence I also evaluated the levels of total STAT3 protein in ustimulated and G-CSF-stimulated neutrophils (Figure 27C-D). Since the amount of total STAT3 was similar in wild type and *Mst1*$^{-/-}$ neutrophils and stable before and after stimulation, I determined that not the total amount but the direct phosphorylation of STAT3 was altered in *Mst1*$^{-/-}$ mice.

5.5.3. CXCR2 expression is differentially regulated in *Mst1*$^{-/-}$ neutrophils under normal conditions and in the bone marrow after G-CSF stimulation

G-CSFR signalling leading to STAT3 phosphorylation triggers additional dimerization and migration of STAT3 to the nucleus to activate the transcription of several genes. CXCR2 is among those STAT3-regulated genes, which expression favours the detachment from the bone marrow and the release of neutrophils to the circulation. In unstimulated mice, the expression level of CXCR2 in circulating *Mst1*$^{-/-}$ neutrophils was decreased compared to wild type neutrophils (Figure 28A-B) even though the expression level of CXCR4, which has the contrary function of CXCR2 in cell attachment, was not different (Figure 28C-D). The stimulation with G-CSF has been reported to directly upregulate the expression of neutrophil CXCR2 (Nguyen-Jackson *et al.*, 2010). In order to see how G-CSF stimulation affected CXCR2 and CXCR4 expression in *Mst1*$^{-/-}$ mice, I collected bone marrow and peripheral blood 24 hours after intravenous injection of NaCl 0.9% (control) or G-CSF (Figure 28E). The expression of CXCR2 in bone marrow wild type neutrophils increased as expected in those mice stimulated with G-CSF but in *Mst1*$^{-/-}$ bone marrow neutrophils this increase was significantly reduced (Figure 28F-G), confirming the impairment in the G-CSFR/STAT3/CXCR2 pathway in Mst1$^{-/-}$ neutrophils. CXCR4 expression decreased as expected in G-CSF stimulated bone

marrow neutrophils but no remarkable differences were appreciated between wild type and *Mst1*$^{-/-}$ mice (Figure 28H-I).

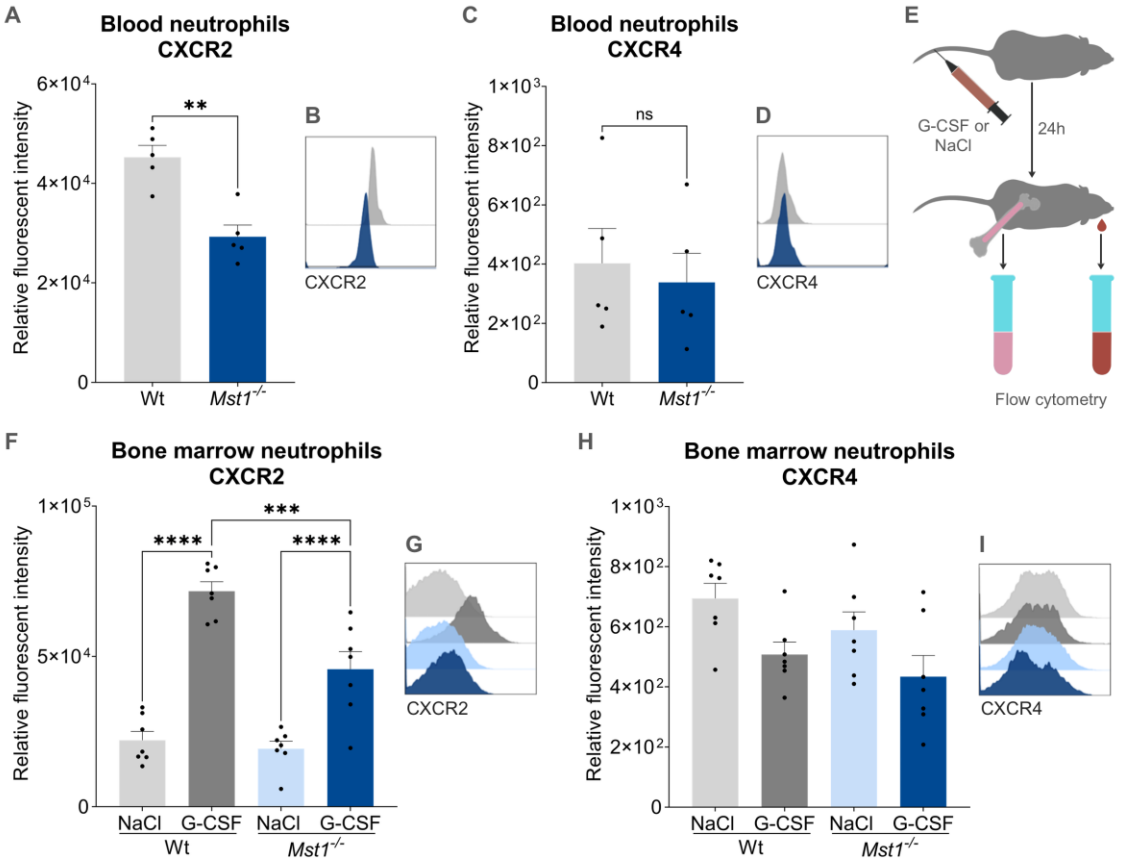

Figure 28. Expression of CXCR2 and CXCR4 under basal conditions and after G-CSF stimulation. (**A-B**) CXCR2 expression on unstimulated circulating neutrophils. Unpaired t-test; **p<0.01; n=5. (**C-D**) CXCR4 expression on unstimulated circulating neutrophils. Unpaired t-test; n=5. (**E**) Schematic representation of bone marrow and blood harvest 24 hours after rmG-CSF intravenous injection. (**F-G**) CXCR2 expression on bone marrow neutrophils of G-CSF or NaCl 0.9% injected mice. Two-way ANOVA; ***p<0.001, ****p<0.0001; n=7. (**H-I**) CXCR4 expression on bone marrow neutrophils of G-CSF or NaCl 0.9% injected mice. Two-way ANOVA; n=7.

5.5.4. Monomeric STAT3 is less available in *Mst1*$^{-/-}$ neutrophils after G-CSF stimulation

In unstimulated cells, STAT3 forms together with other proteins a complex called statosome (Unudurthi *et al.*, 2018). After cell activation STAT3 is released from the complex in a monomeric state and becomes available as substrate of kinases such as JAK2. With intracellular staining for flow cytometry, I was able to detect the expression of cytoplasmic STAT3 as a free monomer, as the epitope that our antibody recognises is blocked for other subunits of the statosome (Figure

29A). By analysing *in vitro* cultured neutrophils, monomeric STAT3 was almost undetectable in unstimulated neutrophils (Figure 29B-C). Nevertheless, STAT3 was clearly detectable in stimulated neutrophils and moreover the levels of STAT3 in *Mst1*$^{-/-}$ neutrophils were significantly diminished (Figure 29B-C), suggesting a role of MST1 in the disassembly of the statosome.

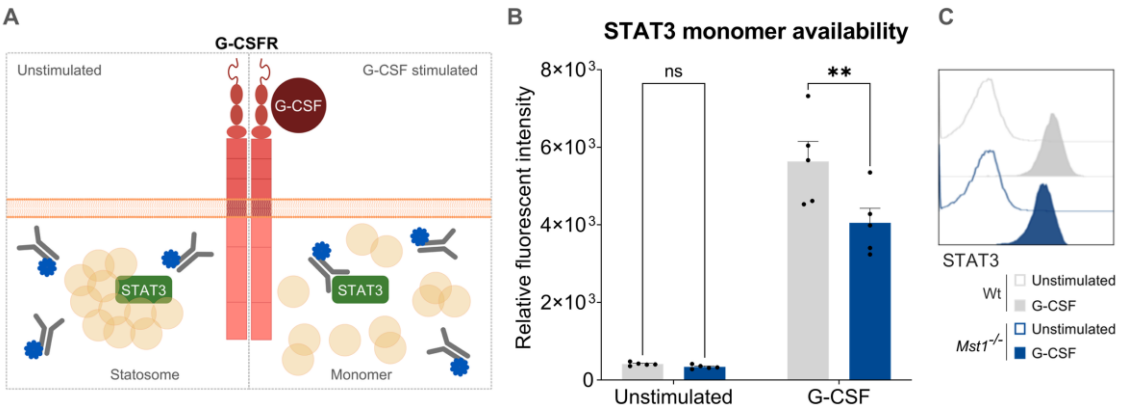

Figure 29. Monomeric STAT3 detection after G-CSF stimulation. (**A**) Scheme of STAT3 antibody recognition in unstimulated and G-CSF stimulated neutrophils. (**B-C**) Relative STAT3 expression in bone marrow unstimulated and 30 minutes stimulated neutrophils. One-way ANOVA; **p<0.01; n=5.

6. Discussion

The central protein of this thesis, mammalian sterile-20 like kinase 1 (MST1), was first described to be the core of a signalling pathway involved in cell growth and proliferation (Reddy and Irvine, 2008). Protein kinases like MST1 allow a quick transmission of external cellular stimuli and its transduction into functional changes in the cell through activation or inhibition of substrates by phosphorylation. However, kinases are promiscuous proteins that interact with various substrates, thus making the whole cell signalling system a complex interconnected net. For instance, MST1 was found to have a major role in proper immune cell trafficking by interacting with proteins other than the ones belonging to its canonical signalling pathway (Nishikimi *et al.*, 2014; Kurz *et al.*, 2016).

MST1 particularly got attention by immunologists after the description of a human combined immunodeficiency syndrome caused by a loss of function mutation in the *Mst1* gene (Abdollahpour *et al.*, 2012; Nehme *et al.*, 2012). In all reported cases of human MST1 deficiency, lymphopenia was the most characteristic sign and efforts were made to understand the role of MST1 in lymphocytes (Mou *et al.*, 2012b; Ueda *et al.*, 2012; Nishikimi *et al.*, 2014). Of note, recurrent acute bacterial and viral infections in those patients combined with transient peripheral neutropenia also pointed towards an important role of MST1 in myeloid cells (Abdollahpour *et al.*, 2012). Therefore, one lab focused on the role of MST1 in neutrophils. We already described that MST1 activates Rab27a effector JFC1 and contributes to the mobilisation of vesicles containing VLA-3, VLA-6, and NE, three proteins necessary for vascular basement membrane penetration, to the cell surface (Kurz *et al.*, 2016). In the study presented here, I demonstrate that MST1 is necessary for the G-CSF-dependent release of neutrophils from the bone marrow into the blood circulation. Using an *in vivo* *Mst1*$^{-/-}$ murine model and *in vitro* approaches I found that the phosphorylation of STAT3 along the G-CSFR signalling pathway is impaired in *Mst1*$^{-/-}$ neutrophils after cell stimulation. Functionally, MST1 directly or indirectly contributes to the availability of monomeric STAT3 in the cytoplasm after cell activation and consequently is essential for the proper G-CSFR-dependent signal transduction in murine neutrophils.

6.1. Characterization of *Mst1*$^{-/-}$ neutrophils

While investigating bone marrow samples, I perceived that *Mst1*$^{-/-}$ samples were always paler and the pellet higher than wild type controls, implying a differential content of leukocytes in the *Mst1*$^{-/-}$ bone marrow. The study of Lee and colleagues in 2018 already evidenced that in bone marrow all hematopoietic stem and progenitor cells except for the megakaryocyte-erythrocyte progenitor had increased pools in *Mst1*$^{fl/fl}$*Mst2*$^{-/-}$*Mx1-Cre* mice (Lee *et al.*, 2018). By counting the absolute number of neutrophils, I found that the neutrophil count in the bone marrow was increased in *Mst1*$^{-/-}$ mice. In some myeloproliferative neoplasms such as chronic neutrophilic leukemia the high proliferation of neutrophils in the bone marrow is also translated to peripheral neutrophilia (Szuber *et al.*, 2019) but *Mst1*$^{-/-}$ mice mice did not have altered counts of circulating neutrophils. Circulating neutrophils that become senescent are cleared mainly by the liver and spleen phagocytes (Rankin, 2010). The content of neutrophils in *Mst1*$^{-/-}$ mice spleen was similar to wild type controls, therefore an enhanced neutrophil clearance that could compensate a possible increased release of neutrophils in the circulation was excluded. For that reason I designed an experimental setup under the hypothesis that *Mst1*$^{-/-}$ neutrophils could have (1) a developmental impairment, (2) enhanced homing to the bone marrow, and/or (3) impaired release to the circulation that could explain the high content of polymorphonucleate cells in the bone marrow but not in the peripheral circulation.

The expression of MST1 on neutrophils had been previously confirmed (Kurz *et al.*, 2016) but since there was no literature on the direct protein expression of MST1 in LSK cells I confirmed it by western blot. The phenotype observed in mice that lack both MST1 and MST2 in the hematopoietic system resembles, albeit in a more exteme version, the phenotype observable in *Mst1*$^{-/-}$ mice (Kurz, Catz and Sperandio, 2018). Because in some cases the absence of a protein leads to the upregulation of its paralogs as a mean of genetic compensation (El-Brolosy and Stainier, 2017), MST2 was analysed and its expression on a protein level was not found although *Mst2* mRNA was present in both wild type and *Mst1*$^{-/-}$ mice. From these results I concluded the effects of MST2 in LSK cells and neutrophils are negligible.

6.2. Maturation and proliferation of *Mst1*$^{-/-}$ neutrophils

Several disorders that involve aberrant neutrophil counts are due to defective maturation of neutrophils. For instance, half of the reported cases of severe congenital neutropenia (SCN) are caused by mutations in the *ELANE* gene that encodes for NE. In those patients, NE mutations cause mistrafficking of the protease that leads to the unfolded protein/ER stress response (Nanua *et al.*, 2011). Since MST1 is also involved in NE trafficking (Kurz *et al.*, 2016), I intended to test whether *Mst1*$^{-/-}$ neutrophils also show maturation defects.

Recently, Evrard and colleagues published a flow cytometry panel that allows the identification of three neutrophil subsets in the bone marrow: pre-neutrophils, immature neutrophils and mature neutrophils (Evrard *et al.*, 2018). By analysing the frequencies of each subset in the bone marrow I wanted to clarify whether a maturation defect in *Mst1*$^{-/-}$ mice could evoke to the accumulation of one of the subsets. However, because the frequencies in wild type and *Mst1*$^{-/-}$ mice were comparable I concluded that there was no maturation defect in *Mst1*$^{-/-}$ neutrophils.

After being released into the circulation, neutrophils continue experiencing sequential changes in an ageing process that leads to their conversion into senescent cells (Borregaard, 2010). Newly generated neutrophils have lower membrane levels of Ly6G (Boivin *et al.*, 2020) and therefore Ly6G expression can also be used as a marker for neutrophil differentiation within the circulation. Interestingly, I observed that *Mst1*$^{-/-}$ circulating neutrophils express less Ly6G than wild type controls, suggesting that *Mst1*$^{-/-}$ circulating neutrophils could be less differentiated.

The aforementioned higher numbers of hematopoietic stem and progenitor cells observed in *Mst1*$^{fl/fl}$*Mst2*$^{-/-}$*Mx1-Cre* mice are caused by an increased proliferation of those cells (Lee *et al.*, 2018). This can be explained by the fact that through its canonical pathway MST1 acts as a tumour suppressor. Accordingly, its absence could be enhancing the proliferation of not only HSPCs but also myeloid progenitors. The BrdU incorporation assay I performed confirmed the increased proliferation in all bone marrow neutrophil maturation subpopulations. With this I concluded that the high numbers of neutrophils in the bone marrow of *Mst1*$^{-/-}$ mice were due to increased proliferation but not defective maturation, and since I

observed normal numbers of *Mst1*$^{-/-}$ circulating neutrophils, enhanced homing ability of peripheral blood neutrophils or a defective response to release stimuli could be reason for the increased number of bone marrow neutrophils.

6.3. Bone marrow homing ability of *Mst1*$^{-/-}$ cells

In order to evaluate the ability of *Mst1*$^{-/-}$ cells to home and engraft host bone marrow, I generated bone marrow chimeric mice. In humans, the success rate of bone marrow transplants in patients carrying mutations in MST1 has shown a 50% success rate while the average success rate of pediatric bone marrow transplants is much higher (Cagdas *et al.*, 2021). From the mice I generated, only three individuals that received wild type donor cells perished due to complications after transplantation. All the rest survived until day 40 post-transplant. Remarkably, the skin opportunistic infection observed in some mice at the end of the experiment was more severe and prevalent in the group of *Mst1*$^{-/-}$ host mice that received *Mst1*$^{-/-}$ donor cells.

In the neutrophil life cycle, circulating neutrophils entering senescence upregulate CXCR4 expression to get retained in CXCL12 rich organs such as the bone marrow. Because neither bone marrow nor circulating *Mst1*$^{-/-}$ neutrophils had differential levels of CXCR4 compared to control wild type neutrophils, *Mst1*$^{-/-}$ bone marrow neutrophils had increased levels of PSGL-1 and integrin β_7, suggesting a possible functional compensation of adhesive properties in circulating neutrophils. Since most leukocytes in the bone marrow are neutrophils (Matsumoto *et al.*, 1995) I intended to evaluate the homing ability of *Mst1*$^{-/-}$ neutrophils by adaptive transfer experiments where I found that both *Mst1*$^{-/-}$ and wild type leukocytes home to the bone marrow at a similar rate. Hence, I conclude that *Mst1*$^{-/-}$ neutrophils do not have enhanced homing abilities that cause the high neutrophil numbers in the bone marrow.

6.4. G-CSF dependent mobilisation of *Mst1*$^{-/-}$ neutrophils

G-CSF is not only the key extracellular regulator of neutrophil production but also essential for the regulation of neutrophil trafficking from the bone marrow to the blood (Semerad *et al.*, 2002). The analysis of serum levels of G-CSF revealed that G-CSF levels are higher in *Mst1*$^{-/-}$ mice both under normal conditions and TNFα-induced inflammation. G-CSF production in mice is tightly regulated by

the IL-23/IL-17 axis (Stark *et al.*, 2005). Tissue resident macrophages and dendritic cells produce IL-23, that influences Th17 cells to produce IL-17 which acts on G-CSF producing cells that release G-CSF to stimulate neutrophil production and release. I speculate that these high levels of G-CSF in *Mst1*$^{-/-}$ mice are due to (1) the imbalanced production of Th17 cells in *Mst1*$^{-/-}$ mice (Geng *et al.*, 2017) combined with (2) the fact that *Mst1*$^{-/-}$ neutrophils fail to extravasate into sites of inflammation (Kurz *et al.*, 2016), so tissue resident phagocytes have less neutrophil debris to phagocytose that would inhibit the IL-23/IL-17 loop (Stark *et al.*, 2005).

The observation of higher G-CSF levels in *Mst1*$^{-/-}$ mice made me wonder how an external stimulation of G-CSF would affect neutrophil trafficking. Interestingly, when assessing peripheral neutrophil counts as means to evaluate the neutrophil functional response to G-CSF, the pronounced neutrophil release that G-CSF stimulation generates in wild type mice was not seen in *Mst1*$^{-/-}$ mice, indicating that *Mst1*$^{-/-}$ mice have an impaired response to G-CSF. In fact, mice that lack the G-CSFR show a similar phenotype of impaired neutrophil mobilisation to the one I observed in *Mst1*$^{-/-}$ mice (Lieschke *et al.*, 1994; Liu *et al.*, 1996; Basu *et al.*, 2000).

To further analyse G-CSF induced bone marrow neutrophil mobilisation I set up *in vivo* imaging of the bone marrow in the mouse skull using multi-photon laser scanning microscopy. Interestingly, *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice had a denser vascular network. Contrary to this observation, MST1 has been reported to promote angiogenesis under hypoxic conditions in a Hippo-pathway independent manner by increasing nuclear import of FOXO1 and therefore regulating the transcription of polarity and migration-associated genes (Kim *et al.*, 2019). Notwithstanding, I presume that the observed denser vascular system is linked to the role of MST1 in organ growth (Reddy and Irvine, 2008). In order to circumvent the differences in the bone marrow microvasculature I performed the analysis in the surrounding area of straight 35-40μm-diameter vessels. The results revealed that less neutrophils in *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice react to G-CSF and migrate within the bone marrow and moreover less neutrophils were recorded intravasating the endothelium and leaving into the blood circulation of *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice, supporting the previous observations of neutrophil release defect. In an effort to

survey the effect of MST1 on the bone marrow endothelium and stromal cells as well, I imaged the neutrophil release from the bone marrow in bone marrow chimeric mice. I observed a tendency of decreased migration of *Mst1*$^{-/-}$*Lyz2*^{eGFP} cells in the *Lyz2*^{eGFP} bone marrow environment compared to *Lyz2*^{eGFP} mice bone marrow, probably not significant due to the sample size-limitation of our study. Most interestingly I observed no difference on the number of intravasated neutrophils between the two chimeric groups that were comparable to the numbers counted in *Mst1*$^{-/-}$*Lyz2*^{eGFP} mice, indicating that indeed MST1 has also an important role on the bone marrow environment to allow the intravasation of neutrophils. G-CSF stimulation has been previously reported to mediate the downregulation of bone marrow CXCL12 expression (Nguyen-Jackson *et al.*, 2010) to promote neutrophil release, therefore a defective G-CSFR signalling in the bone marrow environment could contribute to the defective release of neutrophils.

After the activation of the G-CSFR, the receptor is internalized in endosomes and it can experience two paths: (1) dephosphorylation and deubiquitination that lead to further processing of the receptor in the endoplasmic reticulum and Golgi and recycling to the neutrophil's surface, or (2) the transition to a late endosome and the degradation of the receptor via the lysosome (Dwivedi and Greis, 2017). MST1 could be affecting any of these routes since it has been involved in vesicular trafficking by activating DENND1C, an effector of the trans-Golgi network and recycling regulator of Rab13 (Nishikimi *et al.*, 2014). In addition, MST1 activate JFC1 that together with Rab27a controls the vesicle exocytic pathway in neutrophils (Kurz *et al.*, 2016; Masgrau-Alsina, Sperandio and Rohwedder, 2020). The sustained levels of both total G-CSFR and plasma membrane G-CSFR during G-CSF stimulation of wild type and *Mst1*$^{-/-}$ neutrophils didn't support the hypothesis of an aberrant G-CSFR degradation or recycling pathway.

On myeloid cells, the activation of G-CSFR promotes granulopoiesis through different signalling pathways like JAK-STATs (Tian *et al.*, 1996), ERK/MAPK (Theyab *et al.*, 2021), the PI3K/Akt (Polak and Buitenhuis, 2012), but it has been only been reported to promote neutrophil mobilisation through JAK2-STAT3 (Zhang *et al.*, 2010). The screening of the phosphorylation patterns of the most important proteins in these pathways revealed that STAT3 was underphosphorylated in *Mst1*$^{-/-}$ neutrophils after G-CSF stimulation. Interestingly,

apart from granulopoiesis and neutrophil mobilisation, STAT3 activates the transcription of SOCS3, which is known to block G-CSFR signalling by promoting the degradation of the receptor (Palande *et al.*, 2013), inactivating the kinase activity of JAK proteins (Carow and Rottenberg, 2014) as well as STAT3 activation (Yasukawa *et al.*, 2003) to provide a negative feedback loop and control the expansion of the G-CSFR signalling. In fact, mice lacking STAT3 continue showing normal neutrophil proliferation (Kamezaki *et al.*, 2005) and they even show neutrophilia and a hyperresponsiveness to G-CSF of bone marrow cells (Lee *et al.*, 2002) as a consequence of the absence of the negative loop that SOCS3 provides. Therefore, I understand that without an MST1-dependent STAT3 activation, neutrophils (1) cannot activate the transcription of the machinery that promotes neutrophil release and (2) either the transcription of SOCS3, thus the other pro-granulopoietic pathways activated by the G-CSFR are not negatively regulated.

During G-CSF stimulation, the transcriptional activity of STAT3 upregulates *Il8rb*, the gene that encodes for CXCR2 (Nguyen-Jackson *et al.*, 2010), a receptor fundamental for neutrophil mobilisation. At the same time, STAT3 potentiates downstream signalling of CXCR2 that involves the activation of ERK (Nguyen-Jackson *et al.*, 2010). Aiming to confirm the impaired signalling of the G-CSFR/JAK/STAT3/CXCR2 axis in *Mst1*$^{-/-}$ mice I evaluated the levels of CXCR2 on circulating and bone marrow neutrophils with and without G-CSF stimulation. In unstimulated mice, CXCR2 expression in circulating *Mst1*$^{-/-}$ neutrophils was decreased and so it was in bone marrow neutrophils after G-CSF stimulation. These results imply that the absence of MST1 causes a decrease in G-CSF-dependent CXCR2 mobilisation to the plasma membrane. Since the CXCR2 expression on the neutrophil membrane is decreased but not completely curbed in *Mst1*$^{-/-}$ mice, the absolute higher number of bone marrow neutrophils would compensate the release defect and maintain normal counts of circulating neutrophils. I speculate that this observation could correlate with the intermittent neutropenia observed in human patients (Abdollahpour *et al.*, 2012).

In the neutrophil life cycle, when CXCR2 levels are upregulated CXCR4 levels are downregulated and vice versa. Surprisingly, in *Mst1*$^{-/-}$ neutrophils the levels of CXCR4 were similar to those expressed by wild type neutrophils.

Interestingly, Nguyen-Jackson and colleagues could not relate G-CSF signalling with the downregulation of CXCR4 (Nguyen-Jackson *et al.*, 2010), thus MST1 only affects neutrophil release through its interference with STAT3 in the G-CSFR signalling pathway.

STAT proteins in the steady state instead of being in the cytoplasm as free monomers can be part of multimeric complexes (Ndubuisi *et al.*, 1999; Sehgal, 2008; Unudurthi *et al.*, 2018), that some have named as statosome (Ndubuisi *et al.*, 1999). Upon cell activation, the disassembly of the statosome release STAT proteins in their monomeric form which then can be used as substrate by JAKs and other kinases (Parri *et al.*, 2020) to get activated and modulate the transcription of several genes (Bharadwaj *et al.*, 2020). With the antibody I used for intracellular staining of STAT3 for flow cytometry analysis I could detect STAT3 after wild type neutrophil G-CSF stimulation but not before since the statosome complex prevents recognition of the epitope by STAT3 specific antibody, suggesting that *Mst1*$^{-/-}$ mice have less available STAT3 after cell stimulation while the total amount of STAT3 was similar in *Mst1*$^{-/-}$ and wild type neutrophils. Therefore I propose that mechanistically MST1 is directly or indirectly involved in the disassembly of the statosome and this way contributes to provide the availability of STAT3 monomers for the proper signalling of the G-CSFR pathway.

6.5. Conclusion

In conclusion, I have identified MST1 as an important regulator of the G-CSFR/STAT3/CXCR2 signalling pathway that orchestrates neutrophil mobilization from the bone marrow into the blood circulation and hence regulates neutrophil homeostasis.

In this study, I uncovered that *Mst1*$^{-/-}$ mice have increased numbers of neutrophils in the bone marrow but similar numbers of circulating neutrophils compared to wild type mice. A potential defect in neutrophil differentiation that prevented the neutrophils to fully mature was excluded after examining the neutrophil subsets in the bone marrow. I additionally noticed that serum levels of G-CSF, the major cytokine regulating neutrophil development and mobilisation, were increased in *Mst1*$^{-/-}$ mice under normal conditions as well as during inflammation. Remarkably, the *in vivo* response to G-CSF was defective in *Mst1*$^{-/-}$ mice, since I did not observe the expected neutrophil release to the circulation and

I captured less mobilised and infiltrating neutrophils in the bone marrow of the skull. G-CSF interaction with G-CSFR on neutrophils leads to the activation of several signalling pathways that result in the promotion of granulopoiesis but it is only through JAK2/STAT3 signalling that neutrophil mobilisation is stimulated. I found that STAT3 phosphorylation was diminished in *Mst1*$^{-/-}$ neutrophils after G-CSF stimulation and the impaired transcriptional activity of STAT3 was confirmed through the detection of reduced expression of CXCR2 on unstimulated circulating *Mst1*$^{-/-}$ neutrophils and bone marrow *Mst1*$^{-/-}$ neutrophils after G-CSF stimulation. Finally, I perceived that after cell stimulation the amount of STAT3 available for phosphorylation was diminished in *Mst1*$^{-/-}$ neutrophils, connecting the function of MST1 to the disassembly of the STAT3-containing statosome.

Moreover, these investigations also suggest an important role of MST1 in the endothelial and bone marrow stromal compartment for the proper engraftment of hematopoietic stem cells and the proper release of neutrophils to the circulation.

To conclude, I propose that MST1 intervenes in the G-CSFR signalling pathway by contributing to the disassembly of the statosome and making STAT3 available for the propagation of the signal. The reduced availability and subsequent transcriptional activity of STAT3 causes a diminished expression of pro-mobilisation CXCR2 but at the same time vanishes the negative feedback loop that SOCS3 provides. Therefore *Mst1*$^{-/-}$ neutrophils have an impaired release to the circulation but no developmental malfunction in the bone marrow. These insights expand the ever-growing non-canonical functions of MST1 in immune cells and illustrate the complex role of this kinase in modulating the innate immune system.

7. References

1. Abdollahpour H, Appaswamy G, Kotlarz D, et al. The phenotype of human STK4 deficiency. *Blood*. 2012;119(15):3450-3457. doi:10.1182/blood-2011-09-378158
2. Afzali B, Lombardi G, Lechler RI, Lord GM. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol*. 2007;148(1):32-46. doi:10.1111/j.1365-2249.2007.03356.x
3. Al-Saud B, Alajlan H, Sabar H, et al. STK4 Deficiency in a Patient with Immune Complex Glomerulonephritis, Salt-Losing Tubulopathy, and Castleman's-Like Disease. *J Clin Immunol*. 2019;39(8):823-826. doi:10.1007/s10875-019-00682-9
4. Anderson KL, Smith KA, Conners K, McKercher SR, Maki RA, Torbett BE. Myeloid development is selectively disrupted in PU.1 null mice. *Blood*. 1998;91(10):3702-3710.
5. Avalos BR, Parker JM, Ware DA, Hunter MG, Sibert KA, Druker BJ. Dissociation of the Jak kinase pathway from G-CSF receptor signaling in neutrophils. *Exp Hematol*. 1997;25(2):160-168.
6. Bae SJ, Ni L, Osinski A, Tomchick DR, Brautigam CA, Luo X. SAV1 promotes Hippo kinase activation through antagonizing the PP2A phosphatase STRIPAK. *Elife*. 2017;6:e30278. Published 2017 Oct 24. doi:10.7554/eLife.30278
7. Barge RM, de Koning JP, Pouwels K, Dong F, Löwenberg B, Touw IP. Tryptophan 650 of human granulocyte colony-stimulating factor (G-CSF) receptor, implicated in the activation of JAK2, is also required for G-CSF-mediated activation of signaling complexes of the p21ras route. *Blood*. 1996;87(6):2148-2153.
8. Basu S, Hodgson G, Zhang HH, Katz M, Quilici C, Dunn AR. "Emergency" granulopoiesis in G-CSF-deficient mice in response to Candida albicans infection. *Blood*. 2000;95(12):3725-3733.
9. Bharadwaj U, Kasembeli MM, Robinson P, Tweardy DJ. Targeting Janus Kinases and Signal Transducer and Activator of Transcription 3 to Treat

Inflammation, Fibrosis, and Cancer: Rationale, Progress, and Caution [published correction appears in Pharmacol Rev. 2020 Jul;72(3):605]. *Pharmacol Rev*. 2020;72(2):486-526. doi:10.1124/pr.119.018440

10. Bjerregaard MD, Jurlander J, Klausen P, Borregaard N, Cowland JB. The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood*. 2003;101(11):4322-4332. doi:10.1182/blood-2002-03-0835
11. Boivin G, Faget J, Ancey PB, et al. Durable and controlled depletion of neutrophils in mice. *Nat Commun*. 2020;11(1):2762. Published 2020 Jun 2. doi:10.1038/s41467-020-16596-9
12. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. 2010;33(5):657-670. doi:10.1016/j.immuni.2010.11.011
13. Borroni EM, Mantovani A, Locati M, Bonecchi R. Chemokine receptors intracellular trafficking. *Pharmacol Ther*. 2010;127(1):1-8. doi:10.1016/j.pharmthera.2010.04.006
14. Bruhn KW, Dekitani K, Nielsen TB, Pantapalangkoor P, Spellberg B. Ly6G-mediated depletion of neutrophils is dependent on macrophages. *Results Immunol*. 2015;6:5-7. Published 2015 Dec 21. doi:10.1016/j.rinim.2015.12.001
15. Bunte K, Beikler T. Th17 Cells and the IL-23/IL-17 Axis in the Pathogenesis of Periodontitis and Immune-Mediated Inflammatory Diseases. *Int J Mol Sci*. 2019;20(14):3394. Published 2019 Jul 10. doi:10.3390/ijms20143394
16. Cagdas D, Halacli SO, Tan C, et al. Diversity in Serine/Threonine Protein Kinase-4 Deficiency and Review of the Literature. *J Allergy Clin Immunol Pract*. 2021;9(10):3752-3766.e4. doi:10.1016/j.jaip.2021.05.032
17. Capucetti A, Albano F, Bonecchi R. Multiple Roles for Chemokines in Neutrophil Biology. *Front Immunol*. 2020;11:1259. Published 2020 Jul 9. doi:10.3389/fimmu.2020.01259
18. Carow B, Rottenberg ME. SOCS3, a Major Regulator of Infection and Inflammation. *Front Immunol*. 2014;5:58. Published 2014 Feb 19. doi:10.3389/fimmu.2014.00058

19. Cheng J, Jing Y, Kang D, et al. The Role of Mst1 in Lymphocyte Homeostasis and Function. *Front Immunol*. 2018;9:149. Published 2018 Feb 5. doi:10.3389/fimmu.2018.00149

20. Cheng Y, Ma XL, Wei YQ, Wei XW. Potential roles and targeted therapy of the CXCLs/CXCR2 axis in cancer and inflammatory diseases. *Biochim Biophys Acta Rev Cancer*. 2019;1871(2):289-312. doi:10.1016/j.bbcan.2019.01.005

21. Choi J, Oh S, Lee D, et al. Mst1-FoxO signaling protects Naïve T lymphocytes from cellular oxidative stress in mice. *PLoS One*. 2009;4(11):e8011. Published 2009 Nov 24. doi:10.1371/journal.pone.0008011

22. Corey SJ, Burkhardt AL, Bolen JB, Geahlen RL, Tkatch LS, Tweardy DJ. Granulocyte colony-stimulating factor receptor signaling involves the formation of a three-component complex with Lyn and Syk protein-tyrosine kinases. *Proc Natl Acad Sci U S A*. 1994;91(11):4683-4687. doi:10.1073/pnas.91.11.4683

23. Creasy CL, Chernoff J. Cloning and characterization of a member of the MST subfamily of Ste20-like kinases. *Gene*. 1995;167(1-2):303-306. doi:10.1016/0378-1119(95)00653-2

24. Crequer A, Picard C, Patin E, et al. Inherited MST1 deficiency underlies susceptibility to EV-HPV infections. *PLoS One*. 2012;7(8):e44010. doi:10.1371/journal.pone.0044010

25. Dang TS, Willet JD, Griffin HR, et al. Defective Leukocyte Adhesion and Chemotaxis Contributes to Combined Immunodeficiency in Humans with Autosomal Recessive MST1 Deficiency [published correction appears in J Clin Immunol. 2016 Apr;36(3):336-7]. *J Clin Immunol*. 2016;36(2):117-122. doi:10.1007/s10875-016-0232-2

26. DeKoter RP, Walsh JC, Singh H. PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *EMBO J*. 1998;17(15):4456-4468. doi:10.1093/emboj/17.15.4456

27. Diny NL, Rose NR, Čiháková D. Eosinophils in Autoimmune Diseases. *Front Immunol*. 2017;8:484. Published 2017 Apr 27. doi:10.3389/fimmu.2017.00484

28. Dong Y, Du X, Ye J, et al. A cell-intrinsic role for Mst1 in regulating thymocyte egress. *J Immunol*. 2009;183(6):3865-3872. doi:10.4049/jimmunol.0900678

29. Du X, Shi H, Li J, et al. Mst1/Mst2 regulate development and function of regulatory T cells through modulation of Foxo1/Foxo3 stability in autoimmune disease. *J Immunol*. 2014;192(4):1525-1535. doi:10.4049/jimmunol.1301060

30. Dwivedi P, Greis KD. Granulocyte colony-stimulating factor receptor signaling in severe congenital neutropenia, chronic neutrophilic leukemia, and related malignancies. *Exp Hematol*. 2017;46:9-20. doi:10.1016/j.exphem.2016.10.008

31. Eash KJ, Means JM, White DW, Link DC. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood*. 2009;113(19):4711-4719. doi:10.1182/blood-2008-09-177287

32. El-Brolosy MA, Stainier DYR. Genetic compensation: A phenomenon in search of mechanisms. *PLoS Genet*. 2017;13(7):e1006780. Published 2017 Jul 13. doi:10.1371/journal.pgen.1006780

33. Evrard M, Kwok IWH, Chong SZ, et al. Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity*. 2018;48(2):364-379.e8. doi:10.1016/j.immuni.2018.02.002

34. Fan GH, Lapierre LA, Goldenring JR, Sai J, Richmond A. Rab11-family interacting protein 2 and myosin Vb are required for CXCR2 recycling and receptor-mediated chemotaxis. *Mol Biol Cell*. 2004;15(5):2456-2469. doi:10.1091/mbc.e03-09-0706

35. Fiedler K, Brunner C. The role of transcription factors in the guidance of granulopoiesis. *Am J Blood Res*. 2012;2(1):57-65.

36. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y, Nagata S. Functional domains of the granulocyte colony-stimulating factor receptor. *EMBO J*. 1991;10(10):2855-2865.

37. Furze RC, Rankin SM. Neutrophil mobilization and clearance in the bone marrow. *Immunology*. 2008;125(3):281-288. doi:10.1111/j.1365-2567.2008.02950.x

38. Geng J, Sun X, Wang P, et al. Kinases Mst1 and Mst2 positively regulate phagocytic induction of reactive oxygen species and bactericidal activity. *Nat Immunol*. 2015;16(11):1142-1152. doi:10.1038/ni.3268

39. Geng J, Yu S, Zhao H, et al. Corrigendum: The transcriptional coactivator TAZ regulates reciprocal differentiation of T_H17 cells and T_{reg} cells. *Nat Immunol*. 2017;18(11):1270. doi:10.1038/ni1117-1270c

40. Golub R, Cumano A. Embryonic hematopoiesis. *Blood Cells Mol Dis*. 2013;51(4):226-231. doi:10.1016/j.bcmd.2013.08.004

41. Greenbaum AM, Link DC. Mechanisms of G-CSF-mediated hematopoietic stem and progenitor mobilization. *Leukemia*. 2011;25(2):211-217. doi:10.1038/leu.2010.248

42. Halacli SO, Ayvaz DC, Sun-Tan C, et al. STK4 (MST1) deficiency in two siblings with autoimmune cytopenias: A novel mutation. *Clin Immunol*. 2015;161(2):316-323. doi:10.1016/j.clim.2015.06.010

43. Halder G, Johnson RL. Hippo signaling: growth control and beyond. *Development*. 2011;138(1):9-22. doi:10.1242/dev.045500

44. Hohaus S, Petrovick MS, Voso MT, Sun Z, Zhang DE, Tenen DG. PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol Cell Biol*. 1995;15(10):5830-5845. doi:10.1128/MCB.15.10.5830

45. Horan T, Wen J, Narhi L, et al. Dimerization of the extracellular domain of granuloycte-colony stimulating factor receptor by ligand binding: a monovalent ligand induces 2:2 complexes. *Biochemistry*. 1996;35(15):4886-4896. doi:10.1021/bi9525841

46. Irandoust MI, Aarts LH, Roovers O, Gits J, Erkeland SJ, Touw IP. Suppressor of cytokine signaling 3 controls lysosomal routing of G-CSF receptor [published correction appears in EMBO J. 2007 Jun

6;26(11):2822]. *EMBO J*. 2007;26(7):1782-1793. doi:10.1038/sj.emboj.7601640

47. Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. *J Clin Invest*. 2006;116(5):1218-1222. doi:10.1172/JCI28508

48. Kamezaki K, Shimoda K, Numata A, et al. Roles of Stat3 and ERK in G-CSF signaling. *Stem Cells*. 2005;23(2):252-263. doi:10.1634/stemcells.2004-0173a

49. Katagiri K, Katakai T, Ebisuno Y, Ueda Y, Okada T, Kinashi T. Mst1 controls lymphocyte trafficking and interstitial motility within lymph nodes. *EMBO J*. 2009;28(9):1319-1331. doi:10.1038/emboj.2009.82

50. Katagiri K, Imamura M, Kinashi T. Spatiotemporal regulation of the kinase Mst1 by binding protein RAPL is critical for lymphocyte polarity and adhesion. *Nat Immunol*. 2006;7(9):919-928. doi:10.1038/ni1374

51. Kendrick TS, Bogoyevitch MA. Activation of mitogen-activated protein kinase pathways by the granulocyte colony-stimulating factor receptor: mechanisms and functional consequences. *Front Biosci*. 2007;12:591-607. Published 2007 Jan 1. doi:10.2741/2085

52. Kim YH, Choi J, Yang MJ, et al. A MST1-FOXO1 cascade establishes endothelial tip cell polarity and facilitates sprouting angiogenesis. *Nat Commun*. 2019;10(1):838. Published 2019 Feb 19. doi:10.1038/s41467-019-08773-2

53. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol*. 2014;14(6):377-391. doi:10.1038/nri3667

54. Kliche S, Worbs T, Wang X, et al. CCR7-mediated LFA-1 functions in T cells are regulated by 2 independent ADAP/SKAP55 modules. *Blood*. 2012;119(3):777-785. doi:10.1182/blood-2011-06-362269

55. Knall C, Worthen GS, Johnson GL. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc Natl Acad Sci U S A*. 1997;94(7):3052-3057. doi:10.1073/pnas.94.7.3052

56. Koussounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci Rep*. 2015;5:10775. Published 2015 Jun 8. doi:10.1038/srep10775

57. Kurz AR, Pruenster M, Rohwedder I, et al. MST1-dependent vesicle trafficking regulates neutrophil transmigration through the vascular basement membrane. *J Clin Invest*. 2016;126(11):4125-4139. doi:10.1172/JCI87043

58. Kurz ARM, Catz SD, Sperandio M. Noncanonical Hippo Signalling in the Regulation of Leukocyte Function. *Trends Immunol*. 2018;39(8):656-669. doi:10.1016/j.it.2018.05.003

59. Laslo P, Pongubala JM, Lancki DW, Singh H. Gene regulatory networks directing myeloid and lymphoid cell fates within the immune system. *Semin Immunol*. 2008;20(4):228-235. doi:10.1016/j.smim.2008.08.003

60. Layton JE, Hall NE. The interaction of G-CSF with its receptor. *Front Biosci*. 2006;11:3181-3189. Published 2006 Sep 1. doi:10.2741/2041

61. Lee CK, Raz R, Gimeno R, et al. STAT3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation. *Immunity*. 2002;17(1):63-72. doi:10.1016/s1074-7613(02)00336-9

62. Lee DH, Kim TS, Lee D, Lim DS. Mammalian sterile 20 kinase 1 and 2 are important regulators of hematopoietic stem cells in stress condition. *Sci Rep*. 2018;8(1):942. Published 2018 Jan 17. doi:10.1038/s41598-018-19637-y

63. Levesque JP, Liu F, Simmons PJ, et al. Characterization of hematopoietic progenitor mobilization in protease-deficient mice. *Blood*. 2004;104(1):65-72. doi:10.1182/blood-2003-05-1589

64. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7(9):678-689. doi:10.1038/nri2156

65. Li C, Bi Y, Li Y, et al. Dendritic cell MST1 inhibits Th17 differentiation. *Nat Commun*. 2017;8:14275. Published 2017 Feb 1. doi:10.1038/ncomms14275

66. Lieschke GJ, Grail D, Hodgson G, et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood*. 1994;84(6):1737-1746.

67. Liu F, Wu HY, Wesselschmidt R, Kornaga T, Link DC. Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. *Immunity*. 1996;5(5):491-501. doi:10.1016/s1074-7613(00)80504-x

68. Liu L, McBride KM, Reich NC. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. *Proc Natl Acad Sci U S A*. 2005;102(23):8150-8155. doi:10.1073/pnas.0501643102

69. Lum JJ, Bren G, McClure R, Badley AD. Elimination of senescent neutrophils by TNF-related apoptosis-inducing [corrected] ligand [published correction appears in J Immunol. 2005 Sep 1;175(5):3446]. *J Immunol*. 2005;175(2):1232-1238. doi:10.4049/jimmunol.175.2.1232

70. Lum SH, Bonney D, Cheesman E, Wrignt NB, Hughes S, Wynn R. Successful Curative Therapy With Rituximab and Allogeneic Haematopoietic Stem Cell Transplantation for MALT Lymphoma Associated With STK4-Mutated CD4+ Lymphocytopenia. *Pediatr Blood Cancer*. 2016;63(9):1657-1659. doi:10.1002/pbc.26048

71. Masgrau-Alsina S, Sperandio M, Rohwedder I. Neutrophil recruitment and intracellular vesicle transport: A short overview. *Eur J Clin Invest*. 2020;50(6):e13237. doi:10.1111/eci.13237

72. Matsumoto K, Inukai S, Isaka T, et al. Cell counts in peripheral blood and bone marrow of male C.B-17 scid/scid mice. *Lab Anim*. 1995;29(2):218-222. doi:10.1258/002367795780740230

73. Medvinsky A, Rybtsov S, Taoudi S. Embryonic origin of the adult hematopoietic system: advances and questions. *Development*. 2011;138(6):1017-1031. doi:10.1242/dev.040998

74. Mou F, Praskova M, Xia F, et al. The Mst1 and Mst2 kinases control activation of rho family GTPases and thymic egress of mature thymocytes. *J Exp Med*. 2012;209(4):741-759. doi:10.1084/jem.20111692

75. Nanua S, Murakami M, Xia J, et al. Activation of the unfolded protein response is associated with impaired granulopoiesis in transgenic mice expressing mutant Elane. *Blood*. 2011;117(13):3539-3547. doi:10.1182/blood-2010-10-311704

76. Ndubuisi MI, Guo GG, Fried VA, Etlinger JD, Sehgal PB. Cellular physiology of STAT3: Where's the cytoplasmic monomer?. *J Biol Chem*. 1999;274(36):25499-25509. doi:10.1074/jbc.274.36.25499

77. Nehme NT, Schmid JP, Debeurme F, et al. MST1 mutations in autosomal recessive primary immunodeficiency characterized by defective naive T-cell survival. *Blood*. 2012;119(15):3458-3468. doi:10.1182/blood-2011-09-378364

78. Nerlov C, Graf T. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev*. 1998;12(15):2403-2412. doi:10.1101/gad.12.15.2403

79. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nat Rev Immunol*. 2019;19(4):255-265. doi:10.1038/s41577-019-0141-8

80. Nguyen-Jackson H, Panopoulos AD, Zhang H, Li HS, Watowich SS. STAT3 controls the neutrophil migratory response to CXCR2 ligands by direct activation of G-CSF-induced CXCR2 expression and via modulation of CXCR2 signal transduction. *Blood*. 2010;115(16):3354-3363. doi:10.1182/blood-2009-08-240317

81. Nguyen-Jackson HT, Li HS, Zhang H, Ohashi E, Watowich SS. G-CSF-activated STAT3 enhances production of the chemokine MIP-2 in bone marrow neutrophils. *J Leukoc Biol*. 2012;92(6):1215-1225. doi:10.1189/jlb.0312126

82. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front Cell Infect Microbiol*. 2017;7:373. Published 2017 Aug 25. doi:10.3389/fcimb.2017.00373

83. Nishikimi A, Ishihara S, Ozawa M, et al. Rab13 acts downstream of the kinase Mst1 to deliver the integrin LFA-1 to the cell surface for lymphocyte trafficking. *Sci Signal*. 2014;7(336):ra72. Published 2014 Jul 29. doi:10.1126/scisignal.2005199

84. Nordenfelt P, Tapper H. The role of calcium in neutrophil granule-phagosome fusion. *Commun Integr Biol*. 2010;3(3):224-226. doi:10.4161/cib.3.3.11168

85. Nurieva R, Yang XO, Martinez G, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature*. 2007;448(7152):480-483. doi:10.1038/nature05969

86. Oh S, Lee D, Kim T, et al. Crucial role for Mst1 and Mst2 kinases in early embryonic development of the mouse. *Mol Cell Biol*. 2009;29(23):6309-6320. doi:10.1128/MCB.00551-09

87. Palande K, Meenhuis A, Jevdjovic T, Touw IP. Scratching the surface: signaling and routing dynamics of the CSF3 receptor. *Front Biosci (Landmark Ed)*. 2013;18(1):91-105. Published 2013 Jan 1. doi:10.2741/4089

88. Pan D. The hippo signaling pathway in development and cancer. *Dev Cell*. 2010;19(4):491-505. doi:10.1016/j.devcel.2010.09.011

89. Park UH, Jeong HS, Jo EY, et al. Piperine, a component of black pepper, inhibits adipogenesis by antagonizing PPARγ activity in 3T3-L1 cells. *J Agric Food Chem*. 2012;60(15):3853-3860. doi:10.1021/jf204514a

90. Parri E, Kuusanmäki H, van Adrichem AJ, Kaustio M, Wennerberg K. Identification of novel regulators of STAT3 activity. *PLoS One*. 2020;15(3):e0230819. Published 2020 Mar 31. doi:10.1371/journal.pone.0230819

91. Pelus LM, Fukuda S. Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Exp Hematol*. 2006;34(8):1010-1020. doi:10.1016/j.exphem.2006.04.004

92. Pierelli L, Marone M, Bonanno G, et al. Modulation of bcl-2 and p27 in human primitive proliferating hematopoietic progenitors by autocrine

TGF-beta1 is a cell cycle-independent effect and influences their hematopoietic potential. *Blood*. 2000;95(10):3001-3009.

93. Polak R, Buitenhuis M. The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. *Blood*. 2012;119(4):911-923. doi:10.1182/blood-2011-07-366203

94. Rankin SM. The bone marrow: a site of neutrophil clearance. *J Leukoc Biol*. 2010;88(2):241-251. doi:10.1189/jlb.0210112

95. Reddy BV, Irvine KD. The Fat and Warts signaling pathways: new insights into their regulation, mechanism and conservation. *Development*. 2008;135(17):2827-2838. doi:10.1242/dev.020974

96. Rohwedder I, Kurz ARM, Pruenster M, et al. Src family kinase-mediated vesicle trafficking is critical for neutrophil basement membrane penetration. *Haematologica*. 2020;105(7):1845-1856. doi:10.3324/haematol.2019.225722

97. Roos D. Chronic Granulomatous Disease. *Methods Mol Biol*. 2019;1982:531-542. doi:10.1007/978-1-4939-9424-3_32

98. Sawant KV, Poluri KM, Dutta AK, et al. Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions. *Sci Rep*. 2016;6:33123. Published 2016 Sep 14. doi:10.1038/srep33123

99. Schipp C, Schlütermann D, Hönscheid A, et al. EBV Negative Lymphoma and Autoimmune Lymphoproliferative Syndrome Like Phenotype Extend the Clinical Spectrum of Primary Immunodeficiency Caused by STK4 Deficiency. *Front Immunol*. 2018;9:2400. Published 2018 Oct 16. doi:10.3389/fimmu.2018.02400

100. Sehgal PB. Paradigm shifts in the cell biology of STAT signaling. *Semin Cell Dev Biol*. 2008;19(4):329-340. doi:10.1016/j.semcdb.2008.07.003

101. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2010;2(6):640-653. doi:10.1002/wsbm.86

102. Semerad CL, Liu F, Gregory AD, Stumpf K, Link DC. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the

blood. *Immunity*. 2002;17(4):413-423. doi:10.1016/s1074-7613(02)00424-7

103. Shah M, Patel K, Mukhopadhyay S, Xu F, Guo G, Sehgal PB. Membrane-associated STAT3 and PY-STAT3 in the cytoplasm. *J Biol Chem*. 2006;281(11):7302-7308. doi:10.1074/jbc.M508527200
104. Souza LM, Boone TC, Gabrilove J, et al. Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science*. 1986;232(4746):61-65. doi:10.1126/science.2420009
105. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*. 2005;22(3):285-294. doi:10.1016/j.immuni.2005.01.011
106. Szuber N, Mudireddy M, Nicolosi M, et al. 3023 Mayo Clinic Patients With Myeloproliferative Neoplasms: Risk-Stratified Comparison of Survival and Outcomes Data Among Disease Subgroups. *Mayo Clin Proc*. 2019;94(4):599-610. doi:10.1016/j.mayocp.2018.08.022
107. Theilgaard-Mönch K, Jacobsen LC, Borup R, et al. The transcriptional program of terminal granulocytic differentiation. *Blood*. 2005;105(4):1785-1796. doi:10.1182/blood-2004-08-3346
108. Theyab A, Algahtani M, Alsharif KF, et al. New insight into the mechanism of granulocyte colony-stimulating factor (G-CSF) that induces the mobilization of neutrophils. *Hematology*. 2021;26(1):628-636. doi:10.1080/16078454.2021.1965725
109. Tian SS, Tapley P, Sincich C, Stein RB, Rosen J, Lamb P. Multiple signaling pathways induced by granulocyte colony-stimulating factor involving activation of JAKs, STAT5, and/or STAT3 are required for regulation of three distinct classes of immediate early genes. *Blood*. 1996;88(12):4435-4444.
110. Ueda Y, Katagiri K, Tomiyama T, et al. Mst1 regulates integrin-dependent thymocyte trafficking and antigen recognition in the thymus. *Nat Commun*. 2012;3:1098. doi:10.1038/ncomms2105
111. Unudurthi SD, Nassal D, Greer-Short A, et al. βIV-Spectrin regulates STAT3 targeting to tune cardiac response to pressure overload. *J Clin Invest*. 2018;128(12):5561-5572. doi:10.1172/JCI99245

112. Veldhoen M. Interleukin 17 is a chief orchestrator of immunity. *Nat Immunol*. 2017;18(6):612-621. doi:10.1038/ni.3742

113. Wang S, Dangerfield JP, Young RE, Nourshargh S. PECAM-1, alpha6 integrins and neutrophil elastase cooperate in mediating neutrophil transmigration. *J Cell Sci*. 2005;118(Pt 9):2067-2076. doi:10.1242/jcs.02340

114. Ward AC, Hermans MH, Smith L, et al. Tyrosine-dependent and -independent mechanisms of STAT3 activation by the human granulocyte colony-stimulating factor (G-CSF) receptor are differentially utilized depending on G-CSF concentration. *Blood*. 1999;93(1):113-124.

115. Watari K, Asano S, Shirafuji N, et al. Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with various disorders as estimated by enzyme immunoassay. *Blood*. 1989;73(1):117-122.

116. Wengner AM, Pitchford SC, Furze RC, Rankin SM. The coordinated action of G-CSF and ELR + CXC chemokines in neutrophil mobilization during acute inflammation. *Blood*. 2008;111(1):42-49. doi:10.1182/blood-2007-07-099648

117. Yang J, Chatterjee-Kishore M, Staugaitis SM, et al. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. *Cancer Res*. 2005;65(3):939-947.

118. Yasukawa H, Ohishi M, Mori H, et al. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat Immunol*. 2003;4(6):551-556. doi:10.1038/ni938

119. Yu W, Xu M, Zhang T, Zhang Q, Zou C. Mst1 promotes cardiac ischemia-reperfusion injury by inhibiting the ERK-CREB pathway and repressing FUNDC1-mediated mitophagy. *J Physiol Sci*. 2019;69(1):113-127. doi:10.1007/s12576-018-0627-3

120. Zarebski A, Velu CS, Baktula AM, et al. Mutations in growth factor independent-1 associated with human neutropenia block murine granulopoiesis through colony stimulating factor-1. *Immunity*. 2008;28(3):370-380. doi:10.1016/j.immuni.2007.12.020

121. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A*. 1997;94(2):569-574. doi:10.1073/pnas.94.2.569

122. Zhang H, Nguyen-Jackson H, Panopoulos AD, Li HS, Murray PJ, Watowich SS. STAT3 controls myeloid progenitor growth during emergency granulopoiesis. *Blood*. 2010;116(14):2462-2471. doi:10.1182/blood-2009-12-259630

123. Zhang Y, Foudi A, Geay JF, et al. Intracellular localization and constitutive endocytosis of CXCR4 in human CD34+ hematopoietic progenitor cells. *Stem Cells*. 2004;22(6):1015-1029. doi:10.1634/stemcells.22-6-1015

124. Zhou D, Medoff BD, Chen L, et al. The Nore1B/Mst1 complex restrains antigen receptor-induced proliferation of naïve T cells. *Proc Natl Acad Sci U S A*. 2008;105(51):20321-20326. doi:10.1073/pnas.0810773105

125. Zhu D, Lai Y, Shelat PB, Hu C, Sun GY, Lee JC. Phospholipases A2 mediate amyloid-beta peptide-induced mitochondrial dysfunction. *J Neurosci*. 2006;26(43):11111-11119. doi:10.1523/JNEUROSCI.3505-06.2006

126. Zhu J, Emerson SG. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene*. 2002;21(21):3295-3313. doi:10.1038/sj.onc.1205318

Publications

1. Immler R, Nadolni W, Bertsch A, Morikis V, Rohwedder I, Masgrau-Alsina S, Schroll T, Yevtushenko A, Soehnlein O, Moser M, Gudermann T, Barnea ER, Rehberg M, Simon SI, Zierler S, Pruenster M, Sperandio M. The voltage-gated potassium channel KV1.3 regulates neutrophil recruitment during inflammation. Cardiovasc Res. 2021 Apr 21:cvab133. doi: 10.1093/cvr/cvab133.

2. Rohwedder I, Kurz ARM, Pruenster M, Immler R, Pick R, Eggersmann T, Klapproth S, Johnson JL, Masgrau-Alsina S, Lowell CA, Mócsai A, Catz SD, Sperandio M. Src family kinase-mediated vesicle trafficking is critical for neutrophil basement membrane penetration. Haematologica. 2020 Jul;105(7):1845-1856. doi: 10.3324/haematol.2019.225722.

3. Masgrau-Alsina S, Sperandio M, Rohwedder I. Neutrophil recruitment and intracellular vesicle transport: A short overview. Eur J Clin Invest. 2020 Jun;50(6):e13237. doi: 10.1111/eci.13237.

Affidavit

Masgrau-Alsina, Sergi

Großhadernerstraße 9

82152 Planegg-Martinsried

Germany

I hereby declare, that the submitted thesis entitled

MST1 controls neutrophil homeostasis through regulation of G-CSF receptor signalling

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Planegg-Martinsried, 14 October 2022 — Sergi Masgrau-Alsina

Place, Date — Signature doctoral candidate

Confirmation of congruency between printed and electronic version of the doctoral thesis

Masgrau-Alsina, Sergi

Großhadernerstraße 9

82152 Planegg-Martinsried

Germany

I hereby declare, that the submitted thesis entitled

MST1 controls neutrophil homeostasis through regulation of G-CSF receptor signalling

is congruent with the printed version both in content and format.

Planegg-Martinsried, 14 October 2022 Sergi Masgrau-Alsina

Place, Date Signature doctoral candidate