Studies of granulocyte colony stimulating factor signaling to develop tools for clinical assessments of severe congenital neutropenia

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Abbreviations

AKT	Protein kinase B
BM	Bone marrow
CD	Cluster of differentiation
CNL	Chronic neutrophilic leukemia
CSF	Colony stimulating factor
CSF3R	Colony stimulating factor 3 receptor
ERK	Extracellular signal-regulated kinase
FMO	Fluorescent minus one
G-CSF	Granulocyte colony stimulating factor
HSC	Hematopoietic stem cell
IL	Interleukin
MFI	Median fluorescent intensity
MP	Myeloid progenitor
PAMPs	Pathogen associated molecular patterns
PBL	Peripheral blood leukocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
QB	QuantiBlue
SCN	Severe congenital neutropenia
SEAP	STAT3-inducable secreted embryonic alkaline phosphatase
STAT	Signal transducer and activator of transcription
TLR	Toll like receptor
VUS	Variant of uncertain significance
WT	Wild type

Abstract

Neutropenia is condition characterized by low number of neutrophils in circulation. This leads to an increased risk of infections and is often diagnosed early in life. Extreme cases are known as severe neutropenia and are often associated with inactivating mutations in common neutrophil genes. Granulocyte colony stimulating factor receptor (G-CSFR), encoded by *CSF3R*, is a growth factor receptor known to induce stem cell release from bone marrow (BM), but also stimulate granulopoiesis. Mutations in this gene leading to low neutrophil counts are associated with the disease severe congenital neutropenia 7 (SNC7).

Classification of genetic variants or mutations based solely on sequencing data can be subjective and often lead to misclassification. Pathogenic mutations have the potential to be categorized as variants of uncertain significance (VUS). This also includes mutations in *CSF3R*, where missense mutations can have a large impact on neutrophile production. A functional test to assess the effects of novel missense mutations in the *CSF3R* gene would be beneficial for the diagnostic work up of SCN7 patients.

This project aims to establish cellular assays for the investigation of G-CSF signaling and for functional characterization of *CSF3R* mutations. First, an assay was created using phospho-flow cytometry to study changes in protein signaling after G-CSF stimulation of primary human blood cells. Secondly, a reporter assay was developed for assessing the impact of *CSF3R*-mutations on STAT3 signaling. A specific mutation previously classified as a VUS, p.(Gly27Arg) was also studied to characterize its impacts on STAT3 signaling following receptor stimulation.

Stimulation assays on neutrophils showed a lack of signaling downstream from the G-CSF receptor. We did, however, observe significant signal transduction in the phospho-flow assay for two out of the four signaling proteins (STAT3 and STAT5) when studying monocytes. The reporter-assay was successful in quantifying STAT3 signal after G-CSF stimulation but showed some difficulties with activating mutations. The mutation p.(Gly27Arg) was found to be likely pathogenic with STAT3 signaling barely detectable compared to WT. Both assays created show promising results for clinically validating the significance of CSF3R variants.

1. Introduction

1.1 The immune system

The immune system is the body's defense against any types of infections, cancers, and other foreign substances. It consists of many different types of cells and molecules that work together to stop dangerous or allergenic substances such as bacteria, viruses, or allergens from spreading in the host [1]. The cells and molecules are all specialized for different parts of this process. A key principle of the immune system is its ability to differentiate between invading and native cells [2]. The immune system would attack its own cells without the ability to identify them as native, potentially leading to autoimmune diseases [1]. Conversely, the inability to respond to microbial invaders would lead to chronic infections, a state known as immunodeficiency.

1.1.1 The two arms of the immune system

The immune system may broadly be divided into the innate and the adaptive part, two distinct yet interconnected parts of the immune system (Figure 1.1). The innate immune system acts within hours of infection [3], but is also instrumental for the more cumbersome activation of the adaptive system, a process requiring several days to weeks. Innate immunity is an ancient more general defense system recognizing evolutionary conserved molecular patterns that are present in most invading microbes [4]. The adaptive system on the other hand, can build specialized responses against very specific parts of any invaders. The adaptive system has memory, which contributes to a large diversity and secures a more rapid response upon re-exposure to pathogens [3]. The innate immune system has often been recognized as the memory-less immune system, but newer research has discovered memory in the innate system dubbed trained immunity [5]. Trained immunity involves epigenetic changes, leading to a more enhanced response from the innate immune system during a second exposure from the same agent.

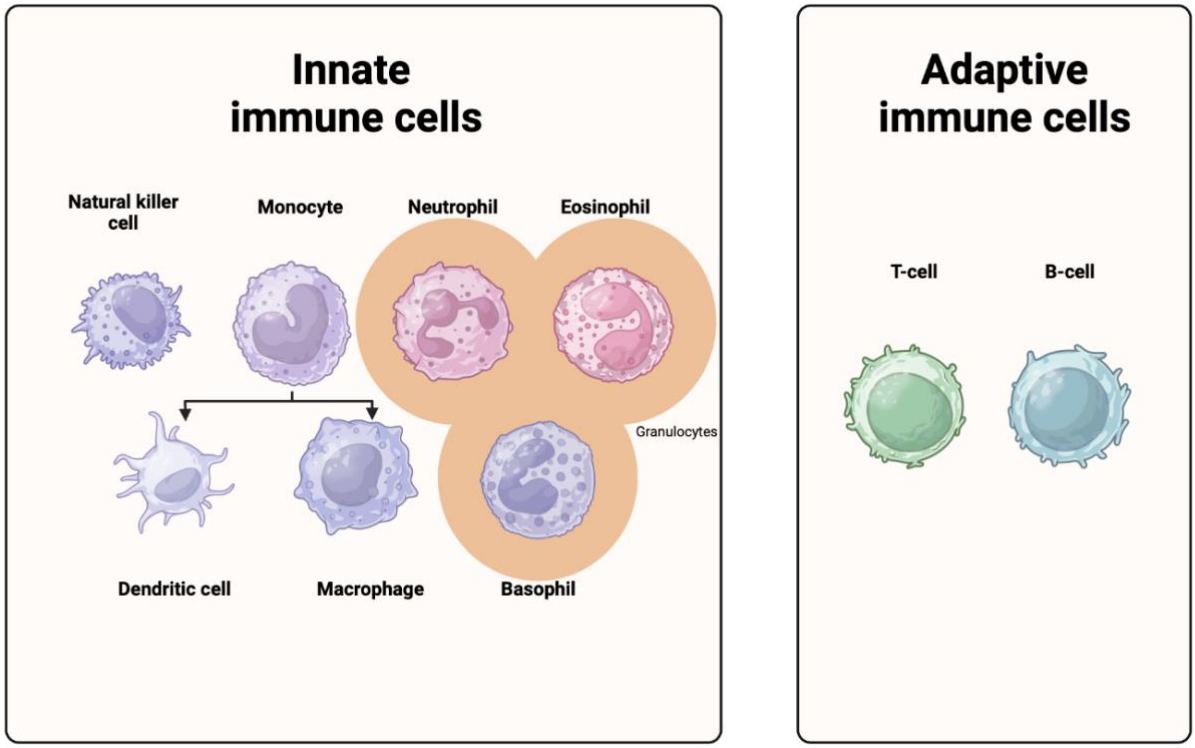

Figure 1.1 – The two arms of the immune system. The cells involved in the innate immune responses are natural killer cell, neutrophils, eosinophils, basophils, and monocytes which differentiates into dendritic cells and macrophages. Relevant for the current study, the granulocytes are highlighted in orange. The adaptive immune system utilizes T-cells and B-cells for its response. Created with BioRender.com.

1.1.2 The innate immune system

An infectious agents first obstacle is physical barriers, such as the ectoderm (i.e., skin) [4, 6]. The ectoderm has different mechanisms for keeping foreign invaders away, such as commensal skin microbes, regulation of pH and anti-microbial peptides/proteins [7]. If successful in crossing the barriers of the ectoderm, an infectious agent would have to deal with both innate and adaptive immunity. Mast cells and dendritic cells (DC) are ready to interact with the infectious agent if it is successful in breaching the dermal layer [8]. They are part of the innate immune system and usually among the first responders to infectious material. Mast cells and DCs recognize molecular patterns present on many of the most common invaders, but not on the hosts own cells, generally known as pathogen associated molecular patterns, or PAMPs [9, 10]. Toll like receptors (TLRs) are key signaling molecules for recognizing these patterns, representing a group of receptors known as pattern-recognition receptors (PRRs) [11]. The first TLR discovered by Beutler and colleagues in 1998 was TLR4, a receptor for lipopolysaccharide endotoxins of gram-negative bacteria [12]. The TLRs can recognize important PAMPs of invading microorganism such as double stranded RNA of

viruses or lipopolysaccharides of the bacterial cell-wall [13]. When a TLR bind to a ligand from a pathogen it starts a signaling cascade that leads to activation of immune cells through the transcription of proinflammatory mediators such as type 1 interferons, cytokines and chemokines [13]. One of the most important processes carried out by these activated immune cells are phagocytosis; the process of capturing and degrading an invading pathogen such as a dangerous bacterium. Phagocytosis serve to kill the pathogen and stops it from spreading throughout the body [14].

Monocytes are the largest blood leukocyte and make up between 5-10% of all leukocytes [15]. They, and eventually other monocyte-derived cells like macrophages and DCs, are capable of phagocytosing pathogens and are important for the innate immune response [16]. The three cell types are also important for the inflammatory response as they produce and excrete cytokines. Finally, the monocyte-derived cells are also important as antigen-presenting cells. They break down antigens into small epitopes that are presented on major histocompatibility complex 2 (MHC II) molecules [17]. The antigen presentation on MHC II is a prerequisite for the activation of B and T cells, making monocytes, macrophages and DCs crucial for activating the adaptive immune system.

1.2 Granulocytes

Granulocytes constitute a major group of leukocytes characterized by multiple granules in their cytoplasm and multi-lobed nuclei, generally split into three lobes. The granules are filled with enzymes and other reactive substances that are released upon activation. Granulocytes are part of the innate immune system, reacting quickly to allergens and pathogens. There are three main types of granulocytic cells, neutrophils, eosinophils, and basophils. Eosinophils are known for their very noticeable granules and have a particular role in the defense against parasitic invasion [18] [2]. The granules contain different signaling molecules and toxic compounds to damage invaders [18]. Basophils are the least abundant white blood cell and are known to play an important role in defending against allergens as they release histamine and heparin in response to allergens [19]. Neutrophils are central for the current thesis and will be presented in the next paragraph.

1.2.1 Neutrophils

Neutrophils are the most abundant leukocyte type present in the blood, constituting up to 70% of all white blood cells in the body [20]. Their multilobed nuclei make them more flexible

when chasing after invaders such as bacteria or viruses [21]. Neutrophils are crucial for innate immunity and for dealing with pathogens quickly during the onset of a possible infection. Neutrophils have granules containing enzymes important for neutrophil mobility, maturation and function [22], and kill invading pathogens in three different ways; Phagocytosis, degranulation, and trapping [23].

Neutrophilic phagocytosis is initiated when receptors present at the cell surface recognize invaders. A phagosome is produced, engulfing the invading agent, and pulls it in a manner similar to endocytosis [14]. The phagosome fuse with granules inside the neutrophil, making the toxic invader accessible for the degrading enzymes present in the granules. Among the mechanisms used to degrade invading substances are reactive oxygen species (ROS) produced by NADPH oxidase [24]. The ROS can activate other degrading enzymes such as proteases or stimulate neutrophil extracellular traps (NETs) production [25]. Another way neutrophils kill pathogens is degranulation [23]. It is the process of exocytosis of granules from the cytoplasm of a neutrophil and into the surrounding environment. Signaling proteins like cytokines trigger degranulation which leads to release of anti-microbial agents [26]. The final way neutrophils kill pathogens is the use of NETs to pin them down [27]. NETs are filaments largely consisting of DNA and histones derived from decondensed chromatin. The chromatin is sticky and act as a blockade hindering invaders from spreading in the tissue. NETs are coated with anti-microbial proteins such as myeloperoxidase which kill invading microorganisms [28].

1.3 Myelopoiesis

The differentiation of hematopoietic stem cells (HSC) to myeloid progenitor cells is known as myelopoiesis, and is one branch of the blood cell production, hematopoiesis [29]. The other branch being lymphopoiesis, production of lymphocytes. Myelopoiesis can further be divided into monopoiesis, production of monocytes and granulopoiesis which will be further explained in the following paragraph. Cytokines are signaling molecules that stimulates many functions of the immune system, including (but not limited to) cell growth, differentiation, or apoptosis [30]. Examples of cytokines are the interleukins (IL), interferons (IFN) or colony stimulating factors (CSF). Signaling through CSFs are important for myelopoiesis

Table **1.1**). Granulocyte colony stimulating factor (G-CSF) is especially important for neutrophil production.

1.3.1 Granulopoiesis

Granulopoiesis is the process where stem cells in the bone marrow (BM) differentiate into granulocytes. A normal person creates around 1 billion neutrophils per kg bodyweight per day [31]. A schematic overview of the granulopoiesis is shown in Figure 1.2. Granulopoiesis starts with a multi-potent hematopoietic stem cell (HSC), known as a hemocytoblast. Potency is a measurement of how many different types a cell can differentiate into [32]. Hemocytoblasts can differentiate into many different cell types and are able to renew themselves [33]. Self-renewal means to be able to go through cell-division while maintaining the same state of potency and staying undifferentiated. During the trajectory of differentiation, the stem- and progenitor cells get more specific and more restricted in terms of which mature states they might develop into. HSCs lose their ability to self-renew as they differentiate into myeloid progenitors (MPs) [34]. Both HCS and MP are multi-potent cells, but whereas HCS can differentiate into all the different types of blood cells, MP can only different into a thrombocyte, basophile, eosinophil, neutrophil, monocyte, mast cell and erythrocyte [35].

As the HSC differentiate into an MP it acquires the ability to express G-CSF receptors in its outer membrane. G-CSF can bind to G-CSF receptors on the MPs and stimulate granulopoiesis [36]. The MP will differentiate into a myeloblast, the first step in the granulopoiesis. Myeloblasts are also known as common myeloid progenitors (CMP) [37]. Further differentiation gives a granulocyte-monocyte progenitor cell (GMP) [38]. GMPs are oligopotent cells as they can differentiate into either granulocytes or monocytes. The cell becomes a granulocyte-progenitor (GP) cell if it stays on the track towards developing into a granulocyte. A GP cell can become any of the three types of granulocytes. Most GP cells differentiate into neutrophils seen as they make up around 70% of all leukocytes [20]. Around 2-3% turn into eosinophils and less than 1% end up as basophils. GP cells go through the stages of promyelocyte, myelocyte, metamyelocyte and banded nucleic granulocyte before ending up as one of the three granulocytes [38]. Granule content varies between the cell states from GP cell to a mature granulocyte.

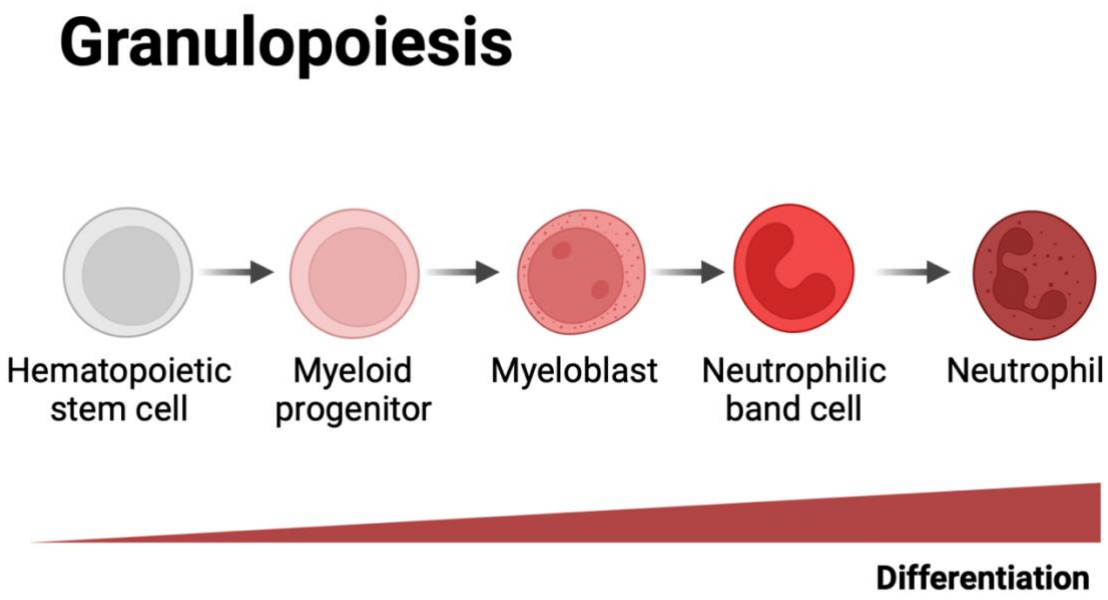

Figure 1.2 – Cell development following the granulopoiesis linage. HSCs differentiate into MP cells that differentiates further into a myeloblast. The myeloblast can differentiate into either a granulocyte or a monocyte but will only end up as a granulocyte following G-CSF signaling. The cell loses potency as it differentiates, meaning that it can develop into fewer different cell types. Adapted from «Cell Differentiation», by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.

Table 1.1 - Colony stimulation factors responsible for differentiation of different cell types, including the cells expressing the receptor and cells producing the ligand.

Ligand	Receptor	Cells expressing receptor	Cells/tissue producing ligand
G-CSF	Colony stimulating factor 3 receptor	Neutrophils, monocytes, stem cells	Endothelial cells, fibroblasts, macrophages
M-CSF	Colony stimulating factor 1 receptor	Monocytes, macrophages, DC, stem cells	Endothelial cells, smooth muscle
GM-CSF	Colony stimulating factor 2 receptor	Monocytes, macrophages, granulocytes, lymphocytes, stem cells	Endothelial cells, T cells, DCs

1.3.2 Granulocyte colony stimulating factor and its receptor

Granulocyte colony stimulating factor 3 (G-CSF) is a cytokine which is fundamentally important for granulopoiesis [36]. It is expressed in response to a variety of different exogenous or endogenous signaling molecules produced during infections or tissue damage such as lipopolysaccharide and vascular endothelial growth factor [39]. While G-CSF is present in the blood in minute to zero concentration under normal conditions [40], the concentration quickly increases during an infection, as G-CSF can be produced by nearly all types of tissue in the body [41].

The G-CSF receptor is a type 1 cytokine receptor responsible for inducing proliferation of neutrophil precursor cells [42], and is known for mobilizing stem cells from bone marrow [43]. G-CSF receptor is also expressed by matured neutrophils and monocytes. G-CSF stimulation enhances neutrophils ability to eliminate pathogens and increases cytokine secretion from monocytes [38].

1.3.3 Structure of the G-CSF receptor

The G-CSF receptor is composed of an extracellular domain, a transmembrane domain, and a cytosolic domain (Figure 1.3). The N-terminus of the protein consists of an Immunoglobulin-like (Ig) C2 domain. Five fibronectin type 3 domains are also present, two of which have been shown to be cytokine receptor homologous (CRH) domains [44]. These are responsible for binding G-CSF and are important for receptor signaling. The cytosolic domain includes three box domains. Box 1 and 2 controls proliferation signaling [45] while Box 3 is essential for regulating cell differentiation [45]. Specific amino acids in the cytosolic domain are acting as docking sites for signaling molecules. In particular, tyrosine at positions 704 (Y704) and 744 (Y744) are important for Janus kinase (JAK) and Signal transducer and activator of transcription (STAT) signaling by serving as docking sites for the signal transducer and activator of transcription 3 (STAT3) transcription factor [45]. Tryptophan 650 (W650) has been shown to act as a docking site necessary for the activation of the JAK2 kinase [46]. Tyrosine 764 (Y764) is responsible for Mitogen-activated protein/Extracellular signal-regulated kinases (MAP/Erk) signaling by binding the adaptor protein Src homology and Collagen (Shc) [47]. A WSXWS-motif is present at the extracellular domain of the receptor. This motif has been found to work as a molecular switch in many cytokine receptors [48]. Binding of the cytokine to the receptor changes the conformation of the WSXWS-motif. The change facilitates dimerization of the receptor and the motif has been found to be very sensitive to mutations [49].

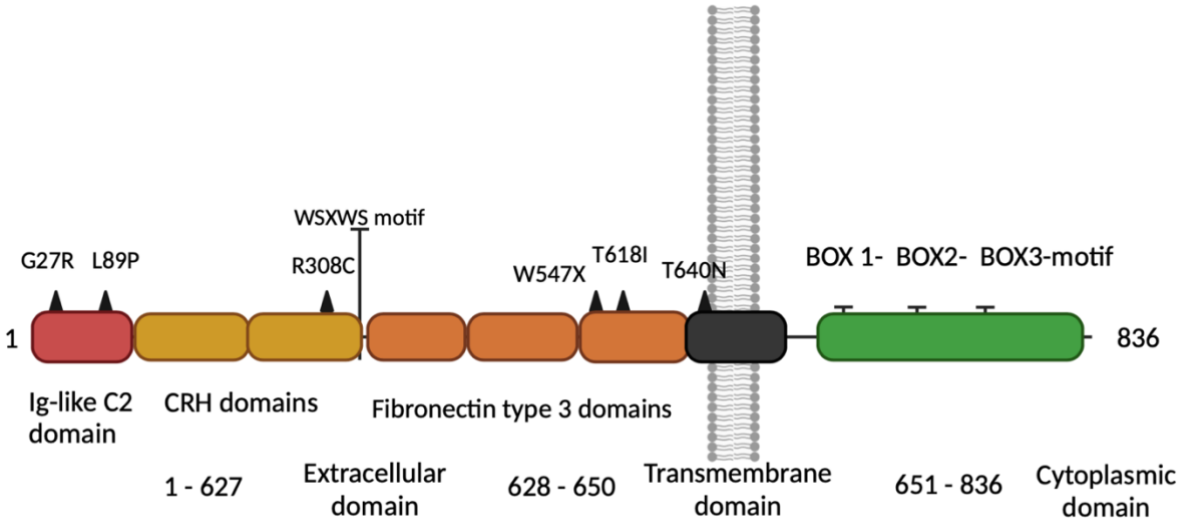

Figure 1.3 – Mutations in the G-CSF. The domains of the receptor are represented with boxes. An Ig-like C2 domain are present at the N-terminal end of the receptor. The two CRH domains present are responsible for ligand-binding which will lead to receptor dimerization. The receptor docks to the cell membrane via its transmembrane domain. Three box-domains are present at the cytosolic domain, each responsible for kinase signaling by binding important signaling molecules. Created with BioRender.com

1.3.4 G-CSF receptor signaling

Kinases are a group of enzymes able to transfer a phosphate group from one molecule to another [50]. The phosphate group is taken from an ATP-molecule and transferred to the target molecule, such as amino acids tyrosine and threonine. Kinase signaling often end up as a signaling cascade with multiple kinases activated downstream of the first activation.
Ligand binding to the G-CSF receptor leads to dimerization of two receptors and activates multiple downstream signaling-pathways involving kinases and transcription factors. The most important ones are JAK/STAT, MAP/Erk and Phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) [45]. The activation of these downstream pathways is responsible for the different effects of G-CSF/G-CSFR signaling as described below. A representation of kinase signaling following G-CSF receptor activation, is shown in Figure 1.4.

JAK/STAT

JAK will bind at the cytoplasmic end of the receptor [51]. Dimerization of the receptors leads to two JAKs phosphorylating and activating each other. The activated JAKs will in turn

phosphorylate tyrosine residues Y704 and Y744 of G-CSFR. A STAT molecule will bind the phosphorylated residues with the help of Src homology 2 (SH2) domains [51]. The STAT-domain is phosphorylated, and the SH2-STAT complex is released from the receptor. Two of these complexes dimerizes and moves into the nucleus where it will bind to DNA and initiate gene expression. STAT3 signaling has been shown to positively impact differentiation of hematopoietic stem cells to granulocytes [45]. STAT5 is important for proliferation and cell survival [52].

MAP/Erk

Tyrosine 764 of G-CSFR is phosphorylated as a result of receptor dimerization [45]. The phosphorylation leads to the binding of growth factor receptor-bound protein 2 (GRB-2) and Son of Sevenless (SOS), initiating a signaling cascade. Moreover, Rat sarcoma virus (Ras) activates Rapidly Accelerated Fibrosarcoma (Raf), which activates Mitogen-activated protein kinase (Mek) and finally Erk. Erk will further activate transcription factors such as MYC which regulates transcription of pro-proliferative genes [53].

PI3K/Akt

Another proliferative signaling molecule is Akt. It is activated through the PI3K pathway which involves phosphorylation of PIP-2 to PIP-3 [54]. PDK1 phosphorylates PIP-3 which allows Akt to bind. Akt is then phosphorylated and activated by mTOR and released from PIP-3. Signaling through Akt is also thought to induce proliferation of myeloid progenitor cells, and hence increasing the number of granulocytes produced [45].

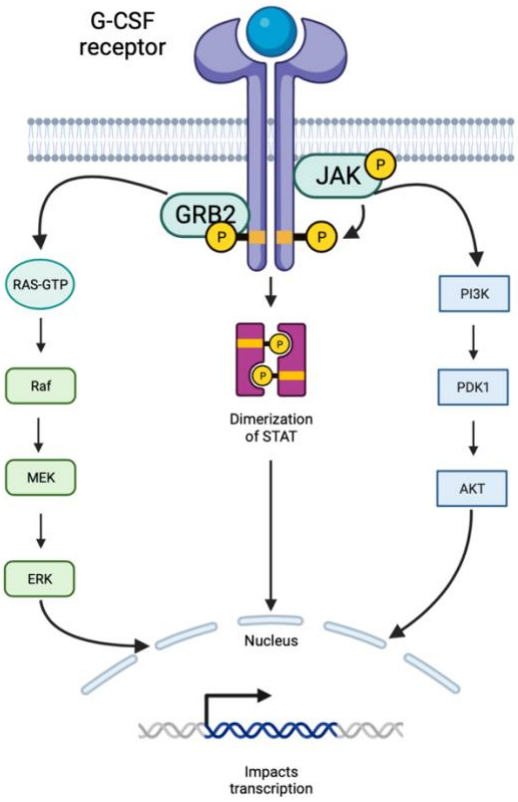

Figure 1.4 – Schematic representation of downstream signaling through Erk, Akt, and STAT after G-CSF binding to its receptor. Ligand binding will lead to dimerization of two G-CSF receptors. **1)** JAK proteins are already bound to tryptophan residues on the cytosolic part of the receptor, two JAKs phosphorylate each other. The activated JAKs phosphorylate tyrosine residues where STAT proteins bind and is also phosphorylated. Two STAT proteins dimerize and moves to the nucleus impacting transcription. **2)** Erk signaling is initiated by phosphorylation of tyrosine residues. This starts a signaling cascade ending with Mek phosphorylating Erk which impacts transcription. **3)** Akt is activated by the PI3K pathway where PIP-2 is phosphorylated to PIP-3, PDK1 adds another phospho-group to PIP-3 which allows Akt to bind and activate, impacting transcription. Created with BioRender.com

1.3.5 Outcomes of G-CSF receptor activation

Stem cell mobilization

G-CSF is known as a potent mediator for mobilizing HSCs and progenitor cells from the bone marrow into the bloodstream [43]. The mechanism underlying how G-CSF stimulate stem cell mobilization is not yet fully understood, but studies have found that G-CSF does not have to be in contact with the cells they mobilize [55], suggesting that there is another mechanism by which G-CSF impacts the stem cells.

HSC are not passively able to move into the blood stream due to several inhibitory factors [43], including Stromal cell-derived factor 1 (SDF1) and vascular cell adhesion molecule-1 (VCAM) [56]. SDF1 is a chemokine, which are small signaling molecules that impact the

movement and migration of leukocytes [57]. SDF1 and VCAM1 levels in the BM have been shown to negatively correlate with neutrophil G-CSF signaling, facilitating increased levels of stem cells entering the blood stream [43, 58]. G-CSF depresses these stem-cell blockers via multiple mechanisms. One of the most studied mechanisms is degradation of SDF1 and VCAM1 by proteolytic cleavage, where proteases like elastase and amino-dipeptidase are released and activated by G-CSF stimulated neutrophils [38, 59].

Neutrophile mobilization

One of the mechanisms by which neutrophils are mobilized towards sites of inflammation is chemotaxis. In this process a cell either moves to an area with higher concentration of chemoattractant molecules, or lower concentration of chemorepellent molecules [60]. The cell must recognize a gradient of attractant for it to be involved in chemotaxis. A chemotactic agent is a molecule that stimulates the chemotactic movement of a cell. Examples of chemotactic agents are lipids like leukotriene B_4, chemokines like neutrophil activating peptide 2 (CXCL7) or interleukin 8 (IL-8) [61]. Chemokine signaling happens through G-protein coupled receptors (GPCRs) which bind the chemotactic agent. The cell will keep moving in one direction when it encounters a chemoattractant molecule leading the cell up the gradient [60].

G-CSF is by itself not a chemotactic agent and cannot mobilize neutrophils directly. Instead, G-CSF is considered a *chemokinetic* agent and has been shown to positively impact migration of neutrophils from circulation into tissue [62]. Chemokinetic indicates that the agent can induce random movements of a cell, chemokinesis. Random movement facilitated by G-CSF increases the likelihood for a neutrophil to enter a chemotactic gradient and to move towards an infected area.

1.4 Fighting an infection

1.4.1 Initiation and clearance

Infections can have fatal consequences for the body, so it is essential that it can defend itself against infectious agents. A potential infectious agent will be recognized by PRRs when entering the body [4]. TLR4 and other receptors will identify PAMPs on the infectant and recognize it as foreign [63]. This will lead to a signaling cascade through cytokines such as G-CSF, IL-6 and interferon gamma (IFN-γ) that will activate specific parts of the immune

system, including important cells of innate immunity such as neutrophils and macrophages [30].

Neutrophils will swarm to the infected area, partly due to the release of G-CSF from macrophages, which positively effects neutrophil mobilization [62]. The neutrophils will fend off the infectant using phagocytosis, degranulation, and trapping [23]. G-CSF secretion will also stimulate granulopoiesis, eventually recruiting more granulocytes into circulation from bone marrow [36].

1.4.2 Resolution

Inflammatory resolution is the process of returning an inflammatory tissue back to homeostasis and involves reducing the number of leukocytes, destroying dead cells and facilitating tissue repair [64]. Neutrophils are the first responders to the site of an infection and will try to neutralize and destroy the infectious agent. Neutrophils are effective but short-lived and will enter apoptosis after around 24 hours [65]. Apoptosis of neutrophils will lead to the release of many dangerous and potentially toxic molecules present in the neutrophil granules. Phagocytosis of dying neutrophils by macrophages limits the potential damage to the tissue surrounding the infected area. Neutrophils undergoing apoptosis have also been shown to regulate granulopoiesis, keeping the neutrophil count at normal levels [66]. Finally, the apoptotic neutrophils also serve as a signal of resolution which will send an anti-inflammatory signal, dampening the immune-response and moving closer to a homeostatic state [67].

1.5 Immunodeficiency

A person that is unable to or has a lowered ability to defend against infections is known to be immune deficient. Broadly speaking, immunodeficiencies are divided into two groups, primary- and secondary-immunodeficiency [68]. Secondary immunodeficiency is also known as acquired immunodeficiency. In secondary immunodeficiency there is an external factor leading to the weakening of the immune system, i.e., infections with the human immunodeficiency virus (HIV), radiation damage or biological drug induced changes. Primary immunodeficiency (PID) implies that no external factors leading to the immunodeficiency exist [42]. Instead, these disorders are caused by pathogenic genetic variants affecting important parts of immunity. People with primary immunodeficiencies are mostly born with them, although clinical manifestations may not become evident until later in

life. The immune system is a complex arrangement of processes so there are many possibilities of potential errors eventually leading to immunodeficiency, some examples are shown in Table 1.2 The intricate and connected parts of the immune system signaling means that one specific error may have a large impact on the entire system. Immunodeficiency is often divided in groups based on what component of the immune system is affected. Many of these groups are representing different cell types, but some are also representing larger molecular systems, such as the complement system.

Mutations affecting B- and T-cells can cause PID by altering antibody production and/or cellular immunity [69]. Deficiency in B-cells have little to no impact on viral infections, but the number of circulating B-cells are often lowered drastically, leading to slower reaction to an infectant. Defects in the T-cells will, on the other hand, affect how the immune system handles a viral infection.

Table 1.2 – Examples of immunodeficiencies by affected components. Possible mutations and effects of the defect are indicated for each immunological component.

Component influenced	Mutation/Defect	Effect	Common infection
T-Cells	Mutated CD3 ε or γ chain gene	T-cell receptor deficiency	Respiratory viral infections
B-Cells	Genes coding for B-cell linker protein or Bruton tyrosine kinase	Lack of B cells	Encapsulated bacterial infection
	Genes coding for light or heavy chain on immunoglobulin	B-cell receptor deficiency	
Neutrophils	G-CSF receptor and HAX1 genes	Neutropenia	Bloodstream infections
Phagocytes	C/EBP	Granule deficiency in neutrophiles	Pyogenic infections
PRR	TLR5 deficiency	Flagellin recognition	*Legionella*
Complement system	Complement protein 1, 2 and 4	Properdin deficiency	*Neisseria meningitidis*

[9, 69-76]

1.5.1 Neutropenia

Neutropenia means low neutrophil numbers and is a chronic condition originating from weakened ability to differentiate HSC into mature functional neutrophils, or an increase in breakdown of neutrophils [77]. Lower neutrophil count leads to a worsened or inadequate response to infections which in turn can increase the risk of significant morbidity or even death. Normal neutrophil counts in blood vary but are often in the ranges of 2500 to 7000 neutrophils per microliter [78]. Neutropenia is divided into three categories based on neutrophil count. Mild, moderate and severe neutropenia is characterized by 1000-1500, 500-1000 and <500 neutrophils per microliter blood, respectively [79]. Severe congenital neutropenia (SCN) is a collective term for multiple types of severe autosomal neutropenia. Mutations at different genes may be responsible for neutropenia, among them are Elastase, Neutrophil Expressed (ELANE), Colony Stimulating Factor 3 Receptor (CSF3-R) and HCLS1 Associated Protein X-1 (HAX1). In addition to recurrent infections, SCN-patients have an increased risk of developing leukemia, chronic caries and gingivitis [80]. Most cases of SCN are treated with recombinant G-CSF. Which increases neutrophil count in circulation, by mobilizing neutrophils from the bone-marrow [81].

1.5.2 Severe congenital neutropenia 7

SCN7 is an autosomal recessive disease associated with mutations in the *CSF3R* gene, encoding the G-CSFR protein (Table 1.3). The mutation(s) leads to a malfunctioning receptor, which in turn stops downstream signaling effects. The mutated receptor impacts the entire myelopoiesis by reduced granulocyte production due to absent G-CSF signaling. Granulopoiesis will then only be initiated via GM-CSF signaling. This halts the proliferation and differentiation of neutrophils as GM-CSF not only facilitates granulocyte differentiation but also differentiation of macrophages [82]. As for all neutropenia, SCN7 is characterized by frequent infections due to low neutrophil count. Patients with SCN7 do not react to G-CSF treatment and thus should be given GM-CSF instead. This defect may have several consequences described below.

1.5.3 Neutrophil recruitment

Tissues in the state of inflammation use various signals such as chemokines and cytokines to recruit neutrophils from circulation to fight invasive agents. Neutrophils need to adhere to the endothelial cells (EC) of the vasculature to be able to move out of the blood stream and into

tissue, and G-CSF signaling plays an important role in this process [83]. The adhering and consecutive migration are completed in a series of events starting with tethering, the initial interaction between ligands on neutrophils and selectins and adhesion molecules on ECs [84]. This binding slows down the neutrophil and starts a rolling process, where the neutrophil keeps moving, but gradually slows down while binding an increased number of selectins. G-CSF is important for this process by facilitating interactions between neutrophils and the adhesion molecule ICAM-1, and is thus an important signaling molecule for recruitment of neutrophils to infected tissue [85].

Patients with reduced G-CSF signaling, such as SCN7 patients, will have reduced adhesion of neutrophils to the ECs. This will in turn lead to fewer neutrophils recruited to the infected tissue. The impact of reduced G-CSF signaling becomes even more prominent when the already reduced number of neutrophils of SCN7 patients are factored in.

1.5.4 Resolution

Inflammatory resolution is important to avoid chronic inflammation [86]. Cytokines, lipids and even the immune cells themselves, in particular neutrophils and macrophages, are important mediators of resolution [64]. Macrophages will in an inflammatory state eat pathogens and release inflammatory cytokines [87]. This state is known as M1 macrophages. M2 macrophages are focused on tissue repair, wound healing, and release anti-inflammatory cytokines, and are therefore crucial for inflammatory resolution. Activated and apoptotic neutrophils release pro-resolution mediators [64]. These mediators signal granulocytes to undergo apoptosis and reduce neutrophil recruitment by downregulating important adhesion molecules. Resolution mediators released from neutrophils will also turn M1 macrophages into M2 macrophages which further enhances the resolution stage by inducing tissue repair. As neutrophil signaling is important for the initiation of inflammatory resolution, patients with reduced neutrophil count, such as SCN7 patients, will not reap the full benefits of this signaling pathway. The severely reduced number of neutrophils can greatly increase the time it takes to move from a state of inflammation to a state of homeostasis and can potentially lead to chronic inflammation.

1.5.5 Periodontitis

Prolonged bacterial infection in the gum can lead to chronic inflammation, a state known as periodontitis, characterized by swelling and retraction of the gum [88]. A prolonged state of

periodontitis can lead to bone loss and eventually loss of teeth. Neutrophils are essential for killing bacteria in the gum, and reduced neutrophil count increases the chance of acquiring periodontitis. Periodontitis is often an early sign of neutropenia, in particular in young neutropenia patients [89]. Periodontitis is otherwise most otherwise prevalent among elderly people. An infant or a child with periodontitis should therefore be checked for leucocyte disorders as it may be a sign of diseases such as neutropenia.

1.5.6 *CSF3R* mutations

Mutations in the *CSF3R* gene may impact the signaling effects of the receptor. As previously explained, the signaling process is complex, requiring interaction of multiple enzymes and signaling molecules to fulfill the desired outcome. A single amino acid change in the receptor can change the properties of that specific receptor-domain. Some of the *CSF3R* mutations are already characterized; p.(Thr640Asn) is known as an activating mutation [90]. The mutation increases receptor-dimerization, which will lead to higher signaling frequencies than wild-type (WT) *CSF3R*. Some areas of the receptor are important for binding of downstream signaling proteins. Y704 is an important amino acid for the JAK-STAT signaling pathway and mutations in this amino acid would reduce signaling through that specific pathway [45]. The p.(Gly27Arg) mutation has previously been interpreted as a variant of uncertain significance (VUS). The mutation is located at the N-terminus of the protein and may impact the structure of the Ig-like C2 domain, which again may affect signaling.
A family where two sisters with congenital neutropenia was investigated for underlying genetic causes at the Department of Medical genetics, Haukeland University Hospital, Bergen, Norway (Figure 1.5). Both sisters with neutropenia were found to be compound heterozygous for the well-known pathogenic mutation p.(Trp547Ter), inherited from the mother, and the VUS p.(Gly27Arg), inherited from the father. The remaining (healthy siblings were heterozygous carriers of one of the mutations. This highlighted the need for new functional tools for clinical assessment of patients with this disease. Understanding the function of the mutation could be beneficial for future diagnostics and therefore the effects of the p.(Gly27Arg) mutation was assessed in the current study.

Table 1.3 – List of mutations associated with SCN7 with nucleotide and amino acid change. Mutations that were selected for mutagenesis in the current study are marked with an x.

Nucleotide sub.	Amino acid change	Function	Tested	Impact
c.79G>A	p.(Gly27Arg)	Unknown	x	Inactivating
c.266T>C	p.(Leu89Pro)	Unknown	x	Inactivating
c.533G>A	p.(Val178Met)	Unknown		Inactivating
c.688C>A	p.(Pro229>His)	Hindering ligand binding		Inactivating
c.922C>T	p.(Arg308>Cys)	Decrease in PM localization	x	Inactivating
c.1640G>A	p.(Trp547Ter)	Premature stop-codon	x	Inactivating
c.1853C>T	p.(Thr618Ile)	Increased dimerization	x	Activating
c.1919C>A	p.(Thr640Asn)	Increased dimerization	x	Activating

[91-94]

PM: Plasma membrane

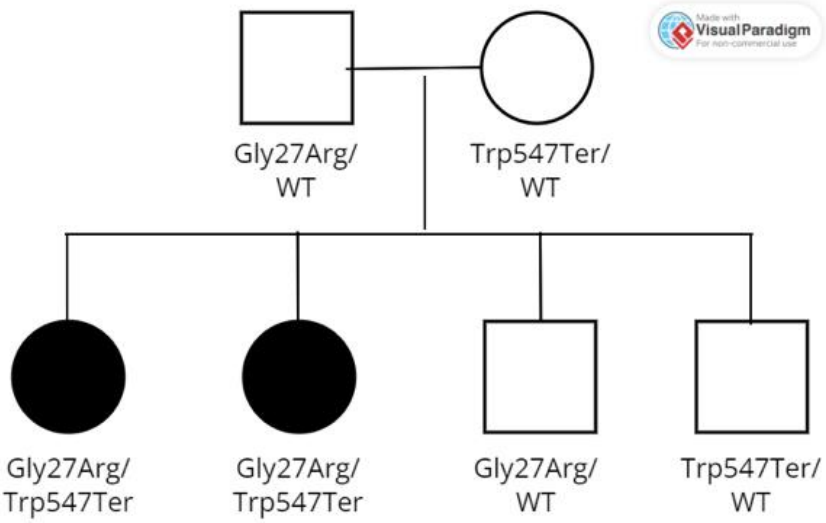

Figure 1.5 – Family tree of a kindred harboring two different CSF3R-mutations. Each parent (both healthy) carries one mutated allele and one WT allele. They have in total four children, two of which have acquired both mutations, while the other two have acquired one mutation and one WT allele. Squares indicate males and circles females. The black filling indicates clinical neutropenia.

1.6 Aims and objectives

Classification of missense mutations in the *CSF3R* gene as pathogenic or benign is challenging and diagnosing SCN7 can be difficult. To aid in diagnosis of SCN7 patients, our aim was to develop functional assays to screen for defects in G-CSFR signaling and to investigate the effects of specific missense mutations. We also aimed at characterizing a specific variant, p.(Gly27Arg), previously described as a VUS.

Specific aims:

1) Establish a phospho-flow assay to assess G-CSFR signaling in patients with suspected SCN7.

2) Investigate and characterize the effects of several *CSF3R* mutations on signaling through the G-CSF receptor in vitro.

2. Materials

2.1 Experimental reagents

Table 2.1 – Primers for site directed mutagenesis. Nucleotide change indicated in bold.

Variant	Direction	Sequence 5´ - 3´	cDNA position 5´- 3´	aa. position 5´- 3´
CSF3R	Forward	CTGGAGGAGTGC**A**GGCACATCAGTG	67 – 92	22 – 30
c.79G>A	Reverse	CACTGATGTGCC**T**GCACTCCTCCAG		
CSF3R	Forward	CTGCTGTTGCTCAA**A**CTGCCTCTGTGG	1906 – 1932	635 – 644
c.1919C>A	Reverse	CCACAGAGGCAGT**T**GAGCAACAGCAG		

aa: amino acid.

Table 2.2 – Primers for sequencing.

Name	Direction	Sequence 5´ - 3´	cDNA position 5´- 3´
CSF3R_1_Fw	Forward	GGGAAACTGCAGCCTGACTT	12 – 31
CSF3R_1_Rev	Reverse	CTGAAGCTCTGCTCCCAGTC	191 – 210
CSF3R_4_Fw	Forward	TGACCAGACTCCATGCCATG	1295 – 1314
CSF3R_6_Rev	Reverse	CAGCGCCTCCATCCCATG	2482 – 2499

Table 2.3 – Sequencing reagents

Name	Manufacturer	Catalog number
BigDye 3.1 Terminator 5x Sequencing Buffer	Applied Biosciences	4336697
BigDye 3.1 ready reaction mix	Applied Biosciences	4336768
Illustra ExoProStar	Cytiva	US77701

Table 2.4 – Flow cytometry reagents

Antibody	Fluorochrome	Isotype	Manufacturer	Catalog number
Anti CD14	PE	Mouse IgG1	Biolegend	367116
Anti CD114	FITC	Mouse IgG1	Miltenyi Biotec	130-120-311
Anti CD163	Biotin conjugated	Mouse IgG1	IQ Products	IQP-570B
Anti pAkt	Alexa Fluor 647	Mouse IgG1	BD biosciences	560343
Anti pSTAT3	Alexa Fluor 647	Mouse IgG2A	BD biosciences	557815
Anti pSTAT5	Alexa Fluor 488	Mouse IgG1	BD biosciences	612598
Anti pErk1/2	Alexa Fluor 488	Mouse IgG1	BD biosciences	612592
Anti CD66b	PE	Mouse IgM	Biolegend	305105
Anti CD89	APC	Mouse IgG1	Biolegend	354105
Anti CD16	BV605	Mouse IgG1	Biolegend	302039
PE Streptavidin	PE		Biolegend	405204

Table 2.5 – Kits used

Name	Manufacturer	Application	Catalog number
QuickChange II	Agilent	Site-directed mutagenesis	200523
Spin Miniprep Kit	Qiagen	Plasmid DNA purification	27104
PureLink HiPure Plasmid Maxiprep Kit	Invitrogen	Plasmid DNA purification	K210007
Quanti-Blue	InvivoGen	STAT3 signaling assay	rep-qbs

Table 2.6 – Stimulants

Stimulant	Signaling protein activated	Catalog number	Manufacturer
IL-6	STAT3	130-095-352	Miltenyi Biotec
IL-2	STAT5	130-097-743	Miltenyi Biotec
PMA (Phorbol 12-myristate 13-acetate)	Erk1/2, Akt	P1585	Merck

Table 2.7 – Lysing, fixation and permeabilization

Name	Application	Manufacturer	Catalog number
Pharm Lyse	Lysing red blood cells	BD Biosciences	555899
Lyse/Fix Buffer 5x	Lysing and fixation	BD Biosciences	558049
Pierce 16% Formaldehyde (w/v)	Fixation	Thermo Scientific	28906
Methanol 99.8%	Permeabilization	Sigma-Aldrich	322415
Ficoll Plaque Plus	Isolate PBMC	Cytiva	17-1440-03

Table 2.8 – Bacterial culture

Name	Manufacturer	Catalog number
LB Broth	BD Biosciences	244620
BD Bacto Agar	BD Biosciences	214050
Blasticidin	InvivoGen	Ant-bl-05
TOP 10 competent cells	Invitrogen	C404010
XL10-GOLD competent cells	Agilent	200314
DH5α competent cells	Thermo Fisher	18258012

Table 2.9 – Cell culturing

Name	Manufacturer	Catalog number
Penicillin Streptomycin	Sigma-Aldrich	P0781
Fetal Bovine Serum (FBS)	Gibco	16000-044
Dulbecco´s Modified Eagle Medium 1 x Glutamax (DMEM)	Gibco	32430-027
RPMI 1640	Gibco	61870-010
HEK293 IL6 reporter cells	Invivogen	hkb-hil6
Phosphate Buffered Saline (PBS)	Gibco	14190-144
QUANTI-Blue	InvivoGen	rep-qbs

Table 2.10 – Primary cells from donors

Blood perseverant	Donor type	Facility
Heparin	Healthy donors	Blood bank Haukeland University Hospital

Table 2.11 – Agarose gel

Name	Manufacturer	Catalog number
10x UltraPure TBE Buffer	InvitroGen	15581-044
GelRed	BioNordika	41003
Agarose	GE Healthcare	17-0554-02
AmpliSize Molecular ruler	Bio rad	1708200

2.2 Instruments and Computer software

Table 2.12 – Instruments

Name	Model	Manufacturer
Thermal Cycler	Veriti 96-Well Fast Thermal Cycler	Applied Biosciences
Plate Reader	SpectraMax Plus 384 Microplate Reader	Molecular Devices
Gel imager	Gel Doc EZ Imager	Biorad
NanoDrop	NanoDrop 2000	Thermo Fisher
Flow cytometer	BD Fortessa	BD Biosciences
Flow cytometer	ID7000	Sony

Table 2.13 – Computer software

Software	Application	Developer
Word (16.69.1)	Thesis writing	Microsoft
Excel (16.70)	Calculations	Microsoft
BioRender	Illustrations	BioRender
Prism9 (9.5.1)	Figures	GraphPad Software
FlowJo (10.8.1)	Flow data analysis	FlowJo LLC
ND-1000 (3.8.1)	DNA concentration measurement	Thermo Scientific
Image Lab (3.0)	Analyzing gel	Bio-Rad
ID7000 software	Flow cytometry	Sony
ApE (3.1.3)	Sequencing work	M. Wayne Davis

3. Methods

3.1 Isolation of cells for *CSF3R* phenotyping

Peripheral blood leucocytes (PBL) were isolated from heathy donor blood to be used in an experiment to phenotype the expression of *CSF3R* on white blood cells. Three different methods were tested to remove the red blood cells (RBC). **1)** Whole blood was mixed with 1X Lysing solution from BD. The blood was vortexed gently and incubated at RT for 15 min protected from light. **2)** BD Pharm-lyse was mixed with blood at a ratio of 2 ml pharm-lyse per 200 µl blood. The mix was vortexed gently and incubated at RT for 15 min protected from light. It was centrifuged 5 min at 200 x g and the supernatant aspirated. The cells were washed twice in 2 ml PBS and centrifuged at 200 x g for 5 min. **3)** BD Lyse/fix buffer was prewarmed to 37°C and added to whole blood at a ratio of 1 volume blood to 20 volumes lysing solution. The cells were vortexed and incubated 10 min at 37°C. The cells were washed twice in 2 ml PBS and centrifuged at 500 x g for 8 min.

3.2 Peripheral blood mononuclear cell (PBMC) isolation

Equal amounts of heparin whole blood and PBS were mixed, 18 ml was pipetted on top of a layer of Ficoll (12 mL). The tube with blood and Ficoll was centrifuged at 400 x g at RT for 30 min with acceleration set to 0 and brake set to 1. The PBMC layer was harvested with a Pasteur pipette and then washed twice with PBS, centrifuged at 300 x g in RT for 7 min. The cells were resuspended in RPMI with 1% FBS at $2x10^6$ cells/ml.

3.3 Whole blood stimulation with G-CSF

For assessing signaling response in granulocytes, healthy donor whole blood was stimulated with G-CSF. Whole blood was prewarmed to 37°C. A total volume of 100 µl blood was stimulated with G-CSF ranging from 0.316 – 100 ng/ml and incubated at 37°C for 15 min. The cells were lysed and fixed using 1x BD Lyse/Fix Buffer. One volume of blood was mixed with 20 volumes of 1x Lyse/Fix at 37°C. The cells were vortexed and incubated in water bath at 37°C for 10 min, before being permeabilized as described in 3.4. The cells were washed twice and centrifuged at 500 x g for 8 min prior to barcoding and antibody staining.

Table 3.1 – Antibody panel for signaling protein staining in whole blood including antibodies, fluorochrome and excitation for each antibody.

Antibody	Fluorochrome	Excitation (nm)	Emission (nm)
Anti-CD66b	PE	561	578
Anti-CD89	APC fire	650	787
Anti-CD16	BV605	405	602
Anti-pSTAT3	Alexa Fluor 647	650	671
Anti-pSTAT5	Alexa Fluor 488	499	520
Anti-pErk1/2	Alexa Fluor 488	499	520
Anti-pAkt	Alexa Flour 647	650	671

3.4 PBMC stimulation

Two million cells in a total volume of 1 ml per stimulant were acclimatized at 37°C for 30 min in FACS tubes. Stimulants were added, and the cells incubated for 15 min at 37°C. The stimulants were added to the following final concentration: 20 ng/ml IL-6, 100U/ml IL-2, 2.5 µl/ml PMA and 20-100 ng/ml G-CSF.

To fixate the cells, paraformaldehyde (PFA) was added for a final concentration of 4% and the cells incubated 15 min at RT. The cells were washed once with PBS and centrifuged 300 x g at RT for 7 min to remove the PFA. The cells were resuspended in the residual volume and incubated on ice for 10 min. For permeabilization, 1 mL ice cold methanol was added while being vortexed and the cells incubated on ice for another 10 min. The cells were washed twice in PBS with centrifugation at 1000 x g and resuspended in 190 µl ice cold PBS for barcoding.

3.5 Barcoding

The dyes Pacific orange and Pacific blue from Thermo Fisher Scientific were used for fluorescent barcoding. Four concentrations of the two colors were chosen, denoted O1-O4 and B1-B4. The concentrations are shown in Table 3.2. Different cell samples were then stained using one concentration of each color. This yielded a four-by-four array of samples shown in Table 3.3. This made it possible to distinguish the different samples from each other in the flow analyzing software. Permeabilized cells were mixed with 4 µL of each concentration at a concentration of $2x10^6$ cells per ml for a total volume of 250 µl. The cells were incubated 30 min on ice in the dark before being washed twice with FACS buffer and centrifuged at 1000 x

g for 5 min at 4°C. The barcode was optimized to be used in a 3 x 4 array, as it best fit with the flow cytometry assays. An example of barcoded cells is shown in Figure 3.1.

Table 3.2 – Barcoding dye concentrations

Color	Concentrations (ng/μL)			
Pacific Orange (O1-O4)	0.125	1	5	20
Pacific Blue (B1-B4)	0.01	0.08	0.4	1.6

Table 3.3 – Barcoding dye pooling

	O1	O2	O3	O4
B1	B1 + O1	B1 + O2	B1 + O3	B1 + O4
B2	B2 + O1	B2 + O2	B2 + O3	B2 + O4
B3	B3 + O1	B3 + O2	B3 + O3	B3 + O4
B4	B4 + O1	B4 + O2	B4 + O3	B4 + O4

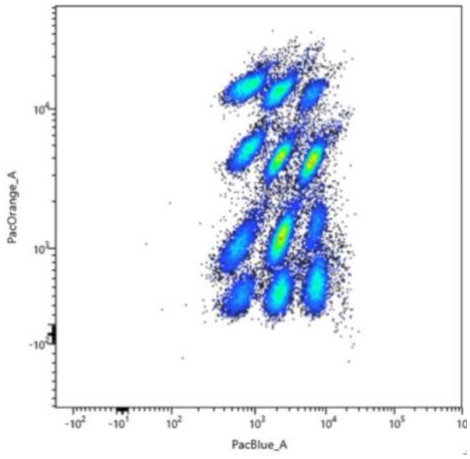

Figure 3.1 – Example of barcoded cells. Permeabilized cells were stained with different concentrations of pacific orange and pacific blue. This staining created colonies of cells that are easy to distinguish on a scatterplot after running flow cytometry. It was decided to use a grid of 3x4 stains as it allowed for running two donors on the same barcode.

3.6 Antibody staining for *CSF3R* phenotyping

An antibody cocktail of CD14 and CD114 was prepared and mixed with the cells. The cells were incubated for 20 min protected from light and washed twice at 1000 x g for 5 min, prior to flow cytometry.

3.7 Antibody staining for monocyte marker titration

PBMC were fixed with PFA and permeabilized using MetOH. Titration of anti-CD163 was performed by staining with a varying amount of CD163 antibody before being stained with a fixed amount of PE-streptavidin. Likewise, to find the optimal amount of PE-streptavidin, PBMC were fixed and stained in the same manner, with 1 µl anti-CD163 and different dilutions of PE-streptavidin: 1:10, 1:30 and 1:100. The samples were run on the Sony ID7000.

3.8 Antibody staining for phosphoflow

Blocking by avidin-block and biotin-block were performed by resuspending cells in 200 µl blocking solution and incubating on ice for 15 min. Cold PBS was added, and the cells centrifuged 1000 x g at 4°C for 5 min. The cells were resuspended at $6x10^6$ cells per ml in FACS buffer. Six tubes of 480 000 cells were prepared and 2.5 µl FC-block was added. The cells were incubated for 10 min on ice before antibody cocktails were added. The cells were incubated with the antibodies for 15 min on ice protected from light, washed with PBS, and resuspended in 95 µl FACS buffer. Five µl 1:30 prediluted streptavidin-PE was added and incubated 15 min on ice protected from light. The cells were washed twice and resuspended in FACS buffer.

3.9 Flow cytometry

One drop of UltraComp eBeads was used to create single-stained controls. One microliter of an anti-body representing each fluorochrome was added to the beads and incubated on ice for 20 min. Unstained control and single stains of the barcoding dyes was also created using unstimulated cells. The flow samples were run on the Sony ID7000 spectral Cell analyzer. Analysis of the flow results were done using FlowJo 10.8.1.

3.10 Cell counting

Cells were counted using C-Chip hemocytometer slides from NanoEnTek. One of the chambers was loaded with 10 µl cell suspension and counted under the microscope. Four grids were counted before the average was calculated. Finally, the cell concentration per mL was calculated using the following formula:

$$\frac{Cells}{ml} = \frac{(cells\ counted\ x\ 10{,}000)}{number\ of\ squares}$$

3.11 Mutagenesis

QuickChange II Site-Directed Mutagenesis Kit from Agilent Technologies was used to perform the mutagenesis. The sample reaction was prepared on ice as follows, 5 μL 10x Reaction buffer, 100 ng dsDNA template (pUNO), 125 ng of primer #1 and #2, 1 μL dNTP mix and ddH_2O to a total of 50 μL. One microliter of PfuUltra (2.5 U/μL) HF DNA polymerase was added and the mix was immediately run through the thermal cycling (Table 3.4) using Veriti 96-Well Fast Thermal Cycler. The PCR product was cooled to < 37°C before 1 μL Dpn 1 (10U/μL) was added to the reaction. The reaction was spun down for 1 min and incubated at 37 °C for 1 h.

Table 3.4 – Thermal cycle parameters of PCR for site directed mutagenesis.

Segment	Cycles	Temperature (°C)	Time
1	1	95	30 sec
2	16	95	30 sec
		55	1 min
		68	1 minute/kb plasmid (6 min)
3	1	68	7 min

3.12 Plasmid preparation in bacteria

LB medium was prepared by mixing 25 g Luria Bertani (LB) with 1 L of ddH_2O. LB agar was prepared by mixing 12.5g of the LB with 7.5 g Bacto Agar and 500 ml ddH_2O. Each solution was autoclaved and stored at 4°C for further use. On the day of agar plate preparation, the agar solution was microwaved until fully dissolved and cooled down to room temperature (RT). Antibiotics was mixed in with the agar for a final concentration of 100 mg/mL, and plates were filled with the agar. Freshly prepared plates were either used for plating of bacterial cultures immediately or stored at 4°C.

Two different bacterial strains were used for transformation as the preferred cell line was not suitable for both DNA isolation methods. One Shot bacterial cells were used for a miniprep, 50 μl was thawed on ice. Either 1.5 μl purified plasmid or 2 μl of mutagenesis reaction was mixed with the cells and mixed gently. The cells were incubated 15 min on ice. A heat shock

was conducted at 42°C in 30 seconds (sec). A total of 250 µl of room tempered SOC medium was added and incubated at 37°C with shaking at 225 rpm for 1 hour (h). Three hundred microliter was spread out on pre-heated agar plates. The plates were incubated overnight (ON) at 37°C.

XL-1 cells were thawed to be used for a maxiprep, 50 µL was allocated to pre-chilled Eppendorf tubes. One microliter of the digested PCR product was added to bacteria and the reaction mixed by swirling. The reaction was incubated on ice for 30 min. The bacteria were heat-shocked for 45 sec at 42°C. The bacteria were incubated on ice for 2 min, and 500 µL preheated SOC medium (42°C) was added. Finally, the reaction was incubated on ice with shaking (225 rpm) for 1h. The entire reaction was plated at an agar plate containing 100 ng/mL Blasticidin. The plates were incubated >16h at 37°C.

LB medium was mixed with Blasticidin for a final concentration of 50 µg/mL at a total volume of 5 ml. Bacteria was collected from the plate and mixed with the medium. The cells were incubated ON at 37°C with shaking (225 rpm).

3.13 DNA isolation

3.13.1 Miniprep

A miniprep was conducted to verify the presence of plasmid after transformation. The QIAprep Spin Miniprep Kit from Qiagen was used for the miniprep. The procedure was conducted in accordance with the protocol that came with the kit, with a few modifications. The elution was done twice, as the first eluate was added back to the column and centrifuged a second time. The last centrifugation was done for 2 min instead of 1.

3.13.2 Maxiprep

A maxiprep was done to extract and purify the plasmids at large quantities. The PureLink HiPure Plasmid Filter Maxiprep Kit from Invitrogen was used for the purification. A colony from the agar plate was mixed in a pre-culture of LB medium and 100 mg/ml blasticidin. It was incubated ON at 37°C and diluted 1:60 in the same growth medium. The maxiprep was conducted in accordance with the protocol in the kit. The concentration of the purified plasmid was checked on a NanoDrop and stored at -20°C.

3.14 Sequencing

The plasmids were sequenced to verify that the mutagenesis was successful, and that the correct mutation was inserted. PCR amplicons flanking the mutations were prepared using plasmid DNA as template, and subsequently sequenced using standard Sanger-based sequencing. The primers for the PCR were diluted to 5 µM with nuclease free water. One microliter mutated DNA was mixed with 2 µL of the diluted forward and reverse primer, 10 µL Amplitaq Gold master mix and 5 µL nuclease free water. The mix was cycled according to Table 3.5.

Table 3.5 – Thermal cycle parameters of PCR for amplification of *CSF3R*.

Segment	Cycles	Temperature (°C)	Time
1	1	94	10 min
2	35	94	30 sec
		58	30 sec
		72	1 minute
3	1	72	7 min

The PCR product was cleaned with illustra ExoProStar 1-Step. A total of 2 µL ExoProStar was added to 5 µL PCR product. The enzyme was activated by heating the solution to 37°C for 15 min and inactivated at 80°C for another 15 min. A forward and a reverse reaction was set up for sequencing. The BigDye Terminator v3.1 Cycle Sequencing Kit was used for the sequencing. Two microliter BigDye 3.1, 2 µL BigDye Buffer (5x), 1 µL primer (5 µM), 1 µL cleaned-up PCR product and 4 µL ddH_2O was mixed and thermal-cycled in accordance with Table 3.6.

Table 3.6 – Thermal cycle parameters of PCR for Sanger sequencing.

Segment	Cycles	Temperature (°C)	Time
1	1	96	1 min
2	25	96	10 sec
		58	5 sec
		60	4 min
3	1	4	hold

Capillary electrophoresis and sequencing were performed by the Sequencing laboratory at the Department of Medical genetics, Haukeland University Hospital.

3.15 Agarose gel and imaging

The specificity of the primers was assessed by running the product of the first PCR on a 1% agarose gel. UltraPure10x TBE buffer from Invitrogen was mixed with 1% agarose and boiled in the microwave until fully dissolved. The solution was cooled to 60°C and 1x GelRed color was added. The gel was set to solidify for 30 min. Biorad AmpliSize Molecular ruler was loaded alongside the PCR products on the gel. The gel was run at 200V, 220mA for 30 min before being imaged using Biorad gel Doc EZ Imager.

3.16 Cell-culturing

IL-6 reporter HEK 293 cells were thawed gently in a 37°C water bath. The cells were moved to a 50 ml centrifuge tube containing 15 ml prewarmed growth medium (DMEM, 4.5 g/L glucose, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS)). The vial was centrifuged at 300 x g for 5 min. The supernatant was removed, and the pellet resuspended in 1 ml and moved to a T25 flask containing 5 mL growth medium. The culture was incubated at 37°C in 5% CO_2.

Medium was swapped twice per week. During the first swap some of the old medium was spun down and a part of the supernatant was readded back to the cells. This was done to keep some of the important growth factors for the cells.

Cells were passaged at around 80% confluency. The cells were loosened and suspended by pipetting. Fresh medium (20 ml) was added to the cells and mixed by pipetting. Out of the total 40 mL, 20 was taken out and put in a new flask. The cells were kept in the appropriate selective medium as provided by the manufacturer of the cell-line. This selective medium was removed before performing transfection. Penicillin streptomycin was added at 10 ml/L.

Stocks of cells at 3 x 10^6 cells/mL were cryopreserved in freezing medium (DMEM with 20% FBS and 10% (v/v) DMSO) in 1 ml. The cells were stored in cryogenic vials at -80°C ON then transferred to -150°C.

3.16.1 Transfection

DMEM without serum or antibiotics was used for the transfection, and 70 000 cells in 150 µl were transferred to the wells of a 96-well plate. The cells reached near 100% confluency in 24

h. Lipofectamine 2000 was used for the transfection, 1 µl transfection agent and 0.5 µg plasmid DNA were diluted separately. The solutions were combined, mixed gently, and incubated at RT for 5 min, before 10 µl of the combined solution was added to each well and incubated 37°C O/N. Controls were transfected using Lipofectamine and DMEM only.

3.16.2 QuantiBlue assay

The cells were stimulated 100 ng/ml G-CSF and 3 ng/ml IL-6 as a positive control and incubated in 37°C O/N. From each well, 20 µl supernatant was transferred to a new plate. Quanti-Blue (QB) reagent and QB buffer were mixed accordingly to the kit. A total of 180 µl of the solution was mixed with the 20 µl supernatant in the new plate and incubated at RT. Optical density at 640 nm of each well was measured using SpectraMax Plus 384 Microplate Reader at 15 min, 1h and 2h.

3.17 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0. A two-way ANOVA (Dunnett) test was conducted when comparing unstimulated samples to samples of different stimulations. The same test was used to compare signaling between WT and variant G-CSF receptor. A significant result would be characterized from an adjusted p-value lower than 0.05.

4. Results

4.1 Validating the presence of G-CSFR in PBL

A flow cytometric staining was conducted to detect G-CSFR in samples of healthy donor blood. The aim was to find which cell types possess the G-CSF receptor. Whole blood was stained with antibodies for CD14 (monocytes) and CD114 (G-CSFR). Three different tests were run to achieve optimal staining in the isolated PBL. BD Lysing solution, Pharm lyse lysing buffer and BD Lyse/fix buffer were all tested as lysing agent. Lyse/fix buffer did not show the desired results and it was decided to work with unfixed cells. BD lysing solution and Pharm lyse were both tested and ultimately Pharm lyse showed the best result. It was used for the validation of G-CSFR in PBL. Gating strategy for the flow analysis is shown in Figure **4.1**.A. BD

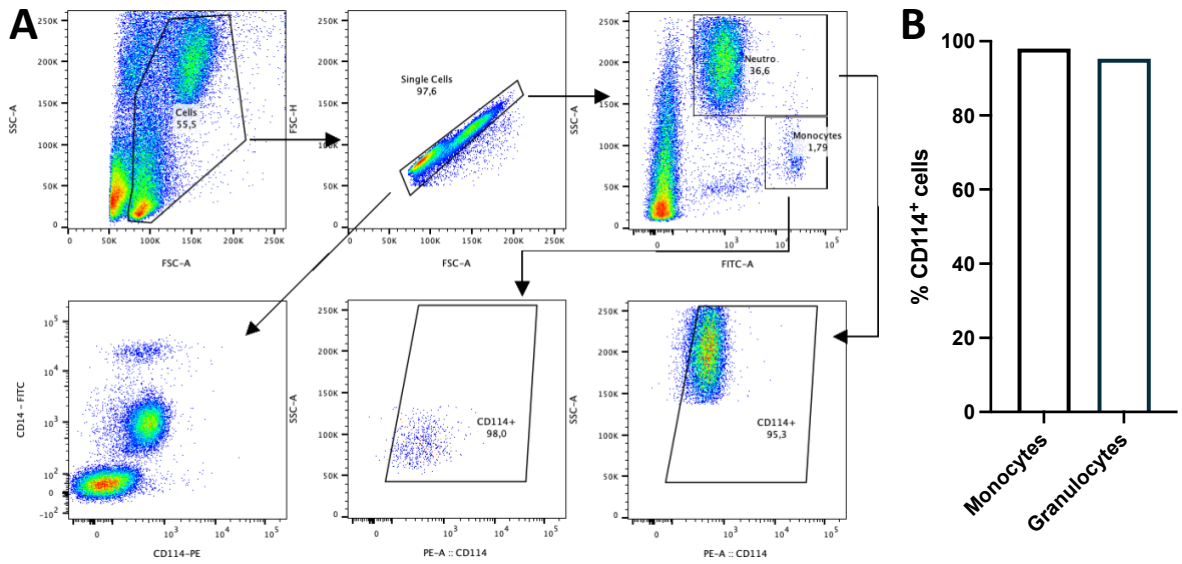

Figure 4.1 – Flow cytometry of PBL to validate the presence of G-CSF receptor. A) The gating strategy used for detecting the receptor. A gate for anti-CD114 (anti-G-CSFR) was established from CD14 fluorescent minus one (FMO) cells. The samples were run on the flow cytometer machine BD LSR Fortessa. B) Percentage of cells positive for the G-CSFR antibody in monocytes and granulocytes. Both populations were close to 100% positive for the receptor.

Both monocytes and neutrophils predominantly expressed the G-CSF receptor with 97.6 and 95.3% positive cells respectively. Both the monocyte- and granulocyte-populations clearly display an increased CD114 expression compared to the remaining cells. This indicates that the G-CSF receptor is present on both monocytes and granulocytes.

4.2 Whole blood G-CSF receptor signaling

Based on receptor expression and abundance, the signaling response in neutrophils was chosen to be used as a marker in an assay to study G-CSF receptor signaling. An antibody panel was set up for analyzing kinase signaling in the form of changes in phosphorylation in whole blood stimulated leukocytes (Table 3.1). The different surface marker antibodies were titrated to achieve the brightest possible signal and the least unspecific binding. Methanol (MetOH) permeabilization is necessary to open the cells to allow the entry of phospho-specific antibodies. MetOH-permeabilization can degrade many important surface receptors used to detect different cell types. A test was then conducted with granulocytes to ensure that it was possible to detect traditional markers like CD16, CD66b or CD89 after MetOH-permeabilization (Figure 4.2). All antibodies tested were compatible with methanol permeabilization, we therefore used CD66b in the following full blood stimulation experiments.

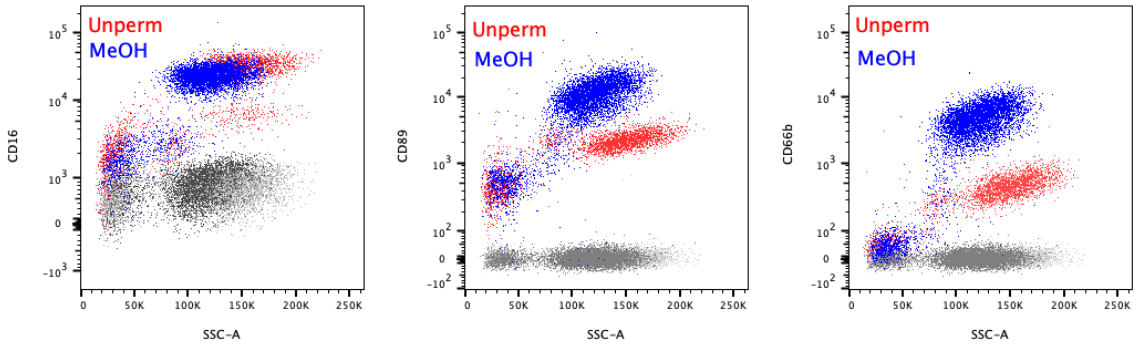

Figure 4.2 – Permeabilization test with CD16, CD89 and CD66b. Unpermeabilized (red) or permeabilized (blue) PBLs were stained with CD16 (A), CD89 (B) or CD66b (C). Two shades of grey are shown representing unstained cells with and without permeabilization.

PBL from healthy controls was stimulated with G-CSF to activate signaling through the G-CSF receptor. The assay was set up to quantify signaling downstream of the receptor. G-CSF was tested at 6 different concentrations ranging from 0.316 – 100 ng/ml. The stimulated blood was stained with CD66b as well as markers for the different signaling proteins phospho-STAT5 Y694, phospho-STAT3 Y705, phospho-Erk1/2 Y204/T202, and phospho-Akt S473 and run on the Sony ID7000. The phospho-antibodies will hereafter be designated pSTAT5, pSTAT3, pErk1/2 and pAkt.

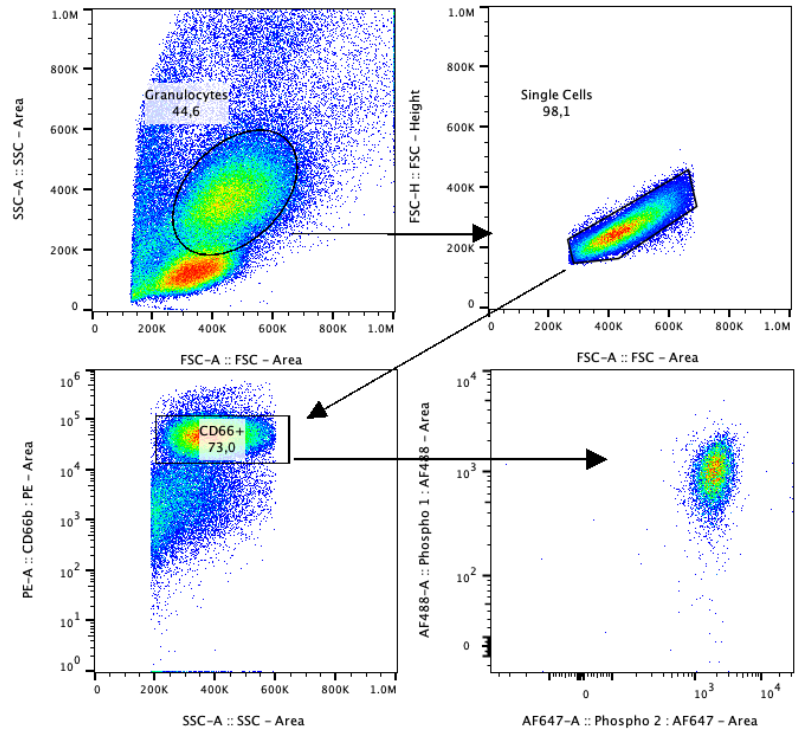

Figure 4.3 – Gating strategy for PBL stimulation experiment with G-CSF. Signaling protein activation was measured in neutrophils using a flow cytometric assay with antibodies for CD66b and relevant signaling proteins.

Table 4.1 – Median florescent intensity values for phosphoantibodies of the four signaling proteins after PBL stimulation with G-CSF. Background intensity has been removed.

Sample	pSTAT5	pSTAT3	pAkt	pERK1/2
Unstained control	382	1262	1262	382
Unstimulated control	541	565	154	572
G-CSF 0.316 ng/ml	678	1234	186	602
G-CSF 1 ng/ml	629	988	334	716
G-CSF 3.16 ng/ml	1225	2402	431	785
G-CSF 10 ng/ml	724	823	285	689
G-CSF 31.6 ng/ml	931	1633	318	734
G-CSF 100 ng/ml	478	797	202	697

Gating strategy is shown in Figure 4.3. Median fluorescent intensity (MFI) values were collected from each population for all the signaling proteins respectively (Table 4.1). Fluorescent minus one (FMO) values, representing unspecific signal from other antibodies

were subtracted. Some changes in MFI values were present, but it was decided to continue working with monocytes as granulocytes did not follow the dose response as expected. The MFI values without removing FMO values are shown in Figure 7.2. in appendix.

4.3 G-CSF signaling in monocytes

4.3.1 Monocyte marker titration

A stimulation-assay using neutrophils to functionally test signaling in SCN7 did not yield the desired results. Monocytes were therefore tested in a similar assay as they too had been shown to express the G-CSF receptor (Figure **4.1**).

CD163 was used as a monocyte marker instead of CD14 as previously used. This was because CD14 is not compatible with MetOH permeabilization. A titration of anti-CD163 was conducted to evaluate CD163 as a monocyte marker in a new antibody panel for PBMC. As anti-CD163 antibody was biotin-conjugated, PE-streptavidin also had to be titrated to be used along with anti-CD163 (Figure 7.4.A in appendix). The optimal amount of antibody would be where the MFI value starts flattening. One microliter was decided to be the right amount and was used for all subsequent experiments. The negative cells (squares) showed a very similar background signal for each amount of antibody, and the increase in MFI from the positive cells (circles) flattened out between 1 and 2 μl.
A similar experiment was set up to find the optimal dilution for PE-streptavidin. The results are presented in Figure 7.4.B in appendix. The dilution 1:100 had the lowest separation between CD163 positive and unstained cells. The 1:30 dilution was chosen for all subsequent experiments as it showed a lower amount of unspecific signal compared to 1:10, while still maintaining a robust MFI signal. The 1:10 dilution displayed the highest signal with anti-CD163 but did also have the highest background signal.

4.3.2 Downstream signaling by Flow Cytometry

In order to study G-CSF receptor downstream signaling, a flow cytometric assay was conducted to validate the presence of signaling protein activity in isolated PBMC. The same panel used for granulocytes (Table 3.1) was used to detect signaling through in monocytes, except that anti-CD66b was swapped with anti-CD163 (see section 4.3.1). PBMCs from healthy donor blood was isolated and stimulated with activators for the different signaling proteins: IL-6 for STAT3, IL-2 for STAT5, PMA for Akt and Erk1/2.

To be able to run multiple stimulants simultaneously, the barcoding protocol (section 3.5) was used. A grid of 3x4 combinations was chosen as it would be enough to run two different donors in the same batch of barcodes. An example of barcoded cells is present in Figure 3.1. Permeabilized cells were therefore barcoded prior to staining with anti-CD163 and antibodies for the different signaling proteins. The samples were run on the ID7000 and analyzed using the FlowJo software. Signaling in the form of increased amount of phosphorylation was evident for all four signaling pathways depending on cell type and stimulation. Figure 4.4 displays increased signaling activity for most signaling proteins in both lymphocytes and monocytes, except for STAT5 which did not show increased signaling in monocytes. CD163-negative cells are referred to as lymphocytes, as lymphocytes constitute the majority of cells in this population.

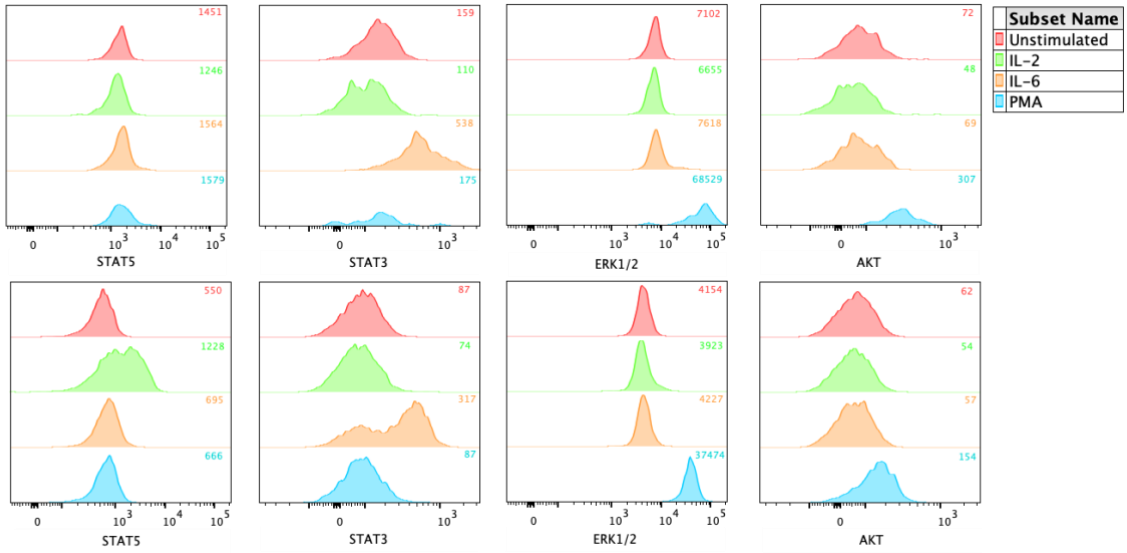

Figure 4.4 – Flow cytometry assay to identify downstream signaling in PBMC. Isolated PBMC was stimulated with IL-6, IL-2 or PMA to activate STAT3, STAT5 and Akt/Erk1/2, respectively. Both monocytes and lymphocytes were stained with phospho-antibodies for the signaling proteins. MFI values are shown for each stimulation.

4.4 Signaling assay for assessing mutations in *CSF3R*

After confirming satisfactory antibody performance, a larger number of donors was used to investigate signaling through G-CSFR. The same flow panel as in section 4.3.2 was used, but this time G-CSF was also added as stimulant at two different concentrations of G-CSF, 20 and 100 ng/ml. The cells were fixated and stained as explained in section 3.4. The samples were

analyzed, and fold change calculated for all signaling proteins within the different donors. The same gating strategy was used for all the samples (Figure 4.5). Representative results from one donor are shown in Figure 4.6.

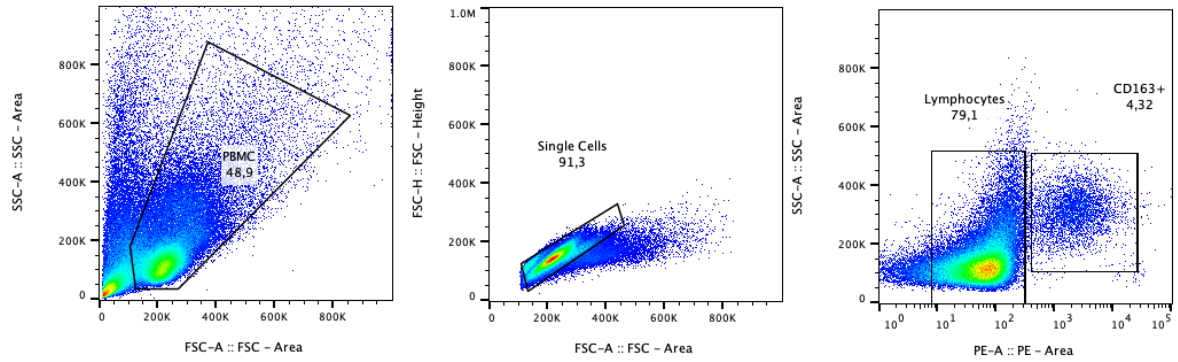

Figure 4.5 – Gating strategy for flow cytometric assay of stimulated PBMC from healthy controls. Dot plots displaying gating strategy for monocytes and lymphocytes analyzed by flow cytometry.

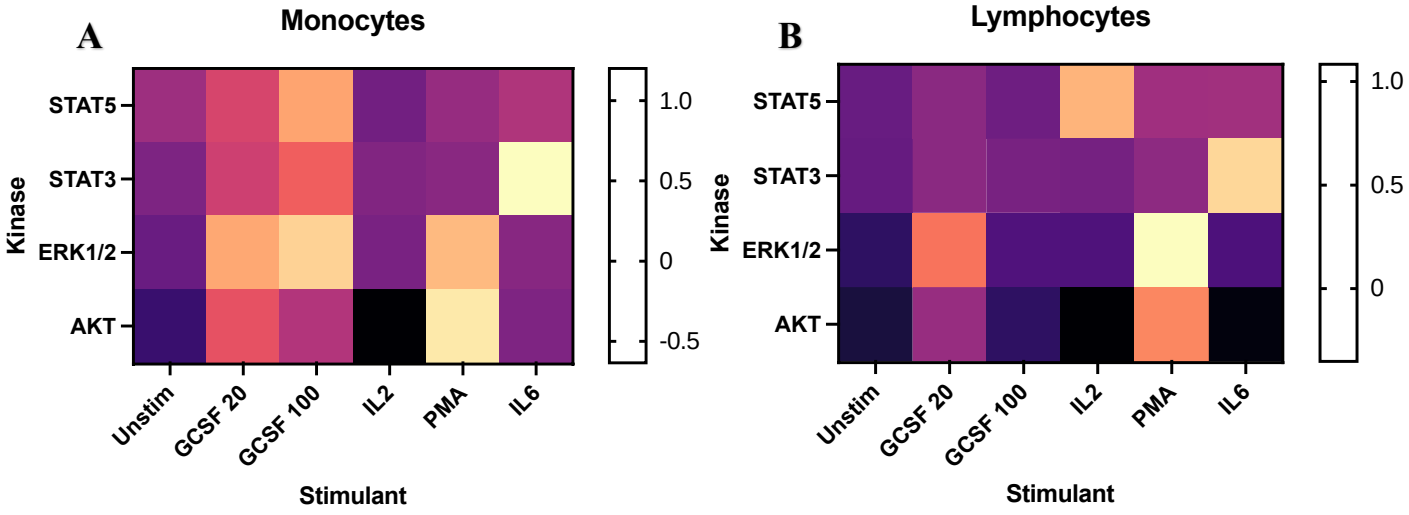

Figure 4.6 – Signaling protein activation in monocytes and lymphocytes. PBMC was stimulated with G-CSF, IL-6, IL-2 and PMA to study signaling protein activation. FMO values from each protein were removed from the measured phospho-signals and fold-change was calculated based on unstimulated cells. The log-transformed fold-change values of one healthy donor are shown as heatmaps for both monocytes (A) and lymphocytes (B).

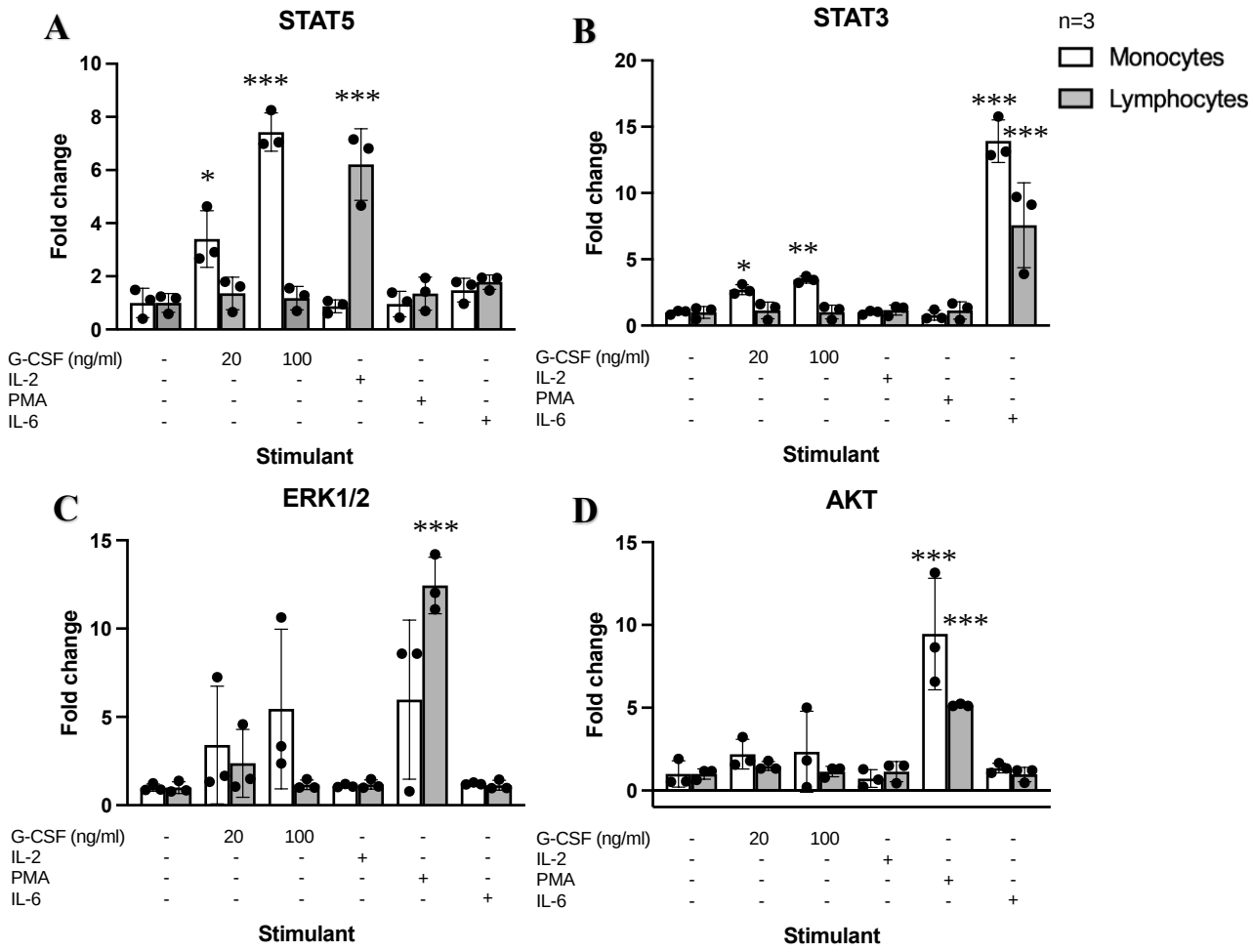

Figure 4.7 – Signaling protein activation after PBMC stimulation with four different stimulants. Column plots displaying phospho-STAT5 Y694 (A), phospho-STAT3 Y705 (B), phospho-Erk1/2 Y204/T202 (C), and phospho-Akt S473 (D) after G-CSF, IL-6, IL-2 or PMA stimulation of PBMC from three healthy donors. FMO values were subtracted from the measured signal and fold change was calculated based on unstimulated cells. Monocytes are shown in white and lymphocytes in grey. The stimulant added are denoted at the bottom of each diagram. *: P-value ≤ 0.05; **: P-value ≤ 0.01; ***: P-value ≤ 0.001.

G-CSF at both 20 and 100 ng/ml activated signaling in monocytes for all the four signaling proteins, although reaching statistically significant changes only for STAT3 and STAT5 (Figure 4.7). Signaling through Erk1/2 and Akt did not reach statistical significance, but high fold change values were still induced in individual samples. Supporting the performance of the assay, all signaling pathways showed activity when stimulated with the appropriate control substances IL-2, IL-6 or PMA. In contrast, all but one protein, Erk1/2, showed close

to no signaling when lymphocytes were stimulated with G-CSF. For monocytes, but not for lymphocytes, increasing the dose of G-CSF from 20 to 100 ng/ml G-CSF seems to increase the signaling of all four proteins.

4.5 Mutagenesis of wild-type *CSF3R*

Two plasmid variants of the *CSF3R* gene were made via site-directed mutagenesis (section 3.11). Transformation, purification, and Sanger sequencing of the plasmids showed successful mutagenesis reactions for both variants (Figure 4.8).

A total of five primer pairs were tested. Gel A (Figure 7.3 in appendix) shows two tested primer pairs, with and without betaine added in the PCR reaction. One clear band are present for PCR products of primer pair, indicated with a white arrow, with a slightly stronger band for the reaction without betaine. The primer pair tested in well 2 and 4 did not yield detectable PCR products for either of the reaction with or without betaine, indicating that the primer pairs did not bind. On gel B, three primer pairs were tested. All three pairs resulted in PCR products giving one dominant band on the gel. All the primer pairs resulting in PCR products with clear bands on the gels were used for PCR amplification of segments of *CSF3R* for sequencing.

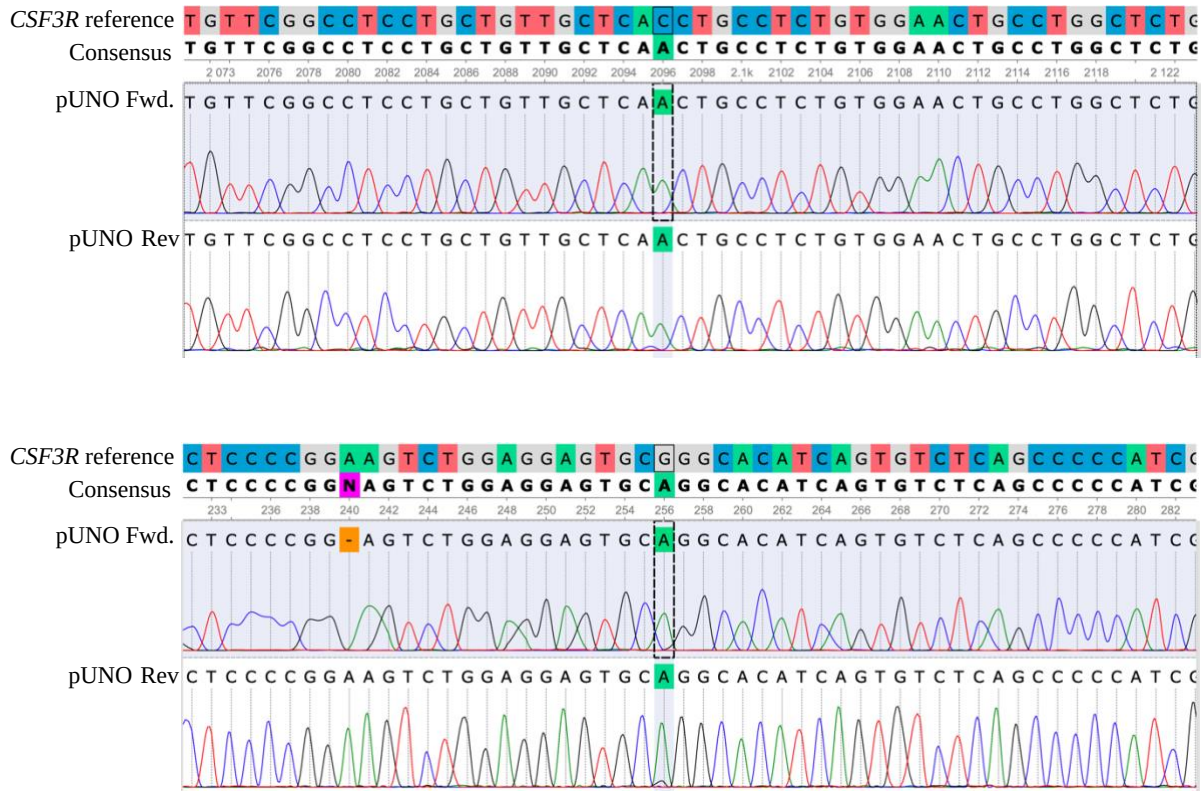

Figure 4.8 – Sequencing data for CSF3R mutations. Variants of *CSF3R* were created using site directed mutagenesis. The plasmids were sequenced bi-directional following mutagenesis. *CSF3R*-reference gene sequence is shown as the top-most sequence. The mutation is highlighted in green and with a black dashed square on the forward reaction. The top-most sequence data are for the p.(Thr640Asn) (c.1919C>A) a substitution from a C to an A are present on both strands. The p.(Gly27Arg) (c.79G>A) mutation is sequenced at the bottom. A substitution from G to an A are present on both strands.

Sanger sequencing of PCR amplicons from mutated plasmids confirmed the presence of desired mutations, and that the mutagenesis had been successful.

4.6 Establishment of a HEK-Blue based reporter system for STAT3 activation

Based on studies of the literature, and from our own data (section 4.4), STAT3 signaling was determined to be a pathway of high significance after G-CSF stimulation. A reporter assay for studying STAT3 signaling in the presence of WT or mutated G-CSFR was therefore designed. In order to quantify STAT3 signaling a commercial reporter cell line developed for detection of bioactive IL-6 was used. The cell line is stably transfected with IL-6 receptor, STAT3 and a STAT3-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Upon IL-6 stimulation, activation of STAT3 is triggered with a subsequent secretion of SEAP into cell-

culture media. IL-6 could then be used as a positive control for the assay. A QuantiBlue solution is added containing a substrate for SEAP. Activity from SEAP leads to a colorimetric change that is measurable through an optical plate reader.
Near 100% confluency was optimal for the transfection and stimulation of the cells. A test was done to determine the number of cells needed to be sown in a 96-well plate to reach close to 100% confluency in 24 h of growth. Cells of varying amount, from 40 000 to 100 000 were seeded into eight wells of a 96-well plate. The confluency after 24 h was noted and the cell count closest to 100% confluency was chosen for stimulation experiments.

Lipofectamine 2000 was used as transfection agent, and an experiment was conducted to determine the optimal amount of lipofectamine to use. The optimal amount would result in highest number of plasmids transfected into the cells without exerting toxic effects affecting cell viability. The cells were transfected as described in section 3.8.1, lipofectamine and plasmid was mixed and added to wells of a 96-well plate at near 100% confluency. The results of the experiment are shown in Figure 4.9.

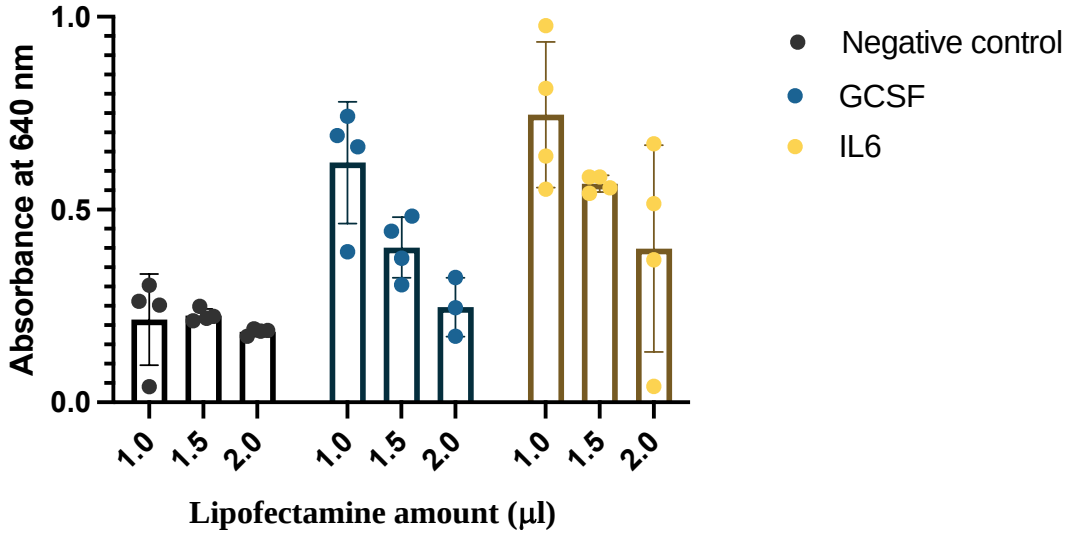

Figure 4.9 – Lipofectamine test to determine optimal transfection reagent. Three different amounts of Lipofectamine were tested to achieve optimal transfection of HEK-Blue IL-6 reporter cells. Plasmids with G-CSF receptor was transfected into the cells, G-CSF and IL-6 was used as stimulant. STAT3 signaling could then be detected and IL-6 could be used as a positive control and unstimulated cells were used as negative control. Absorbance was measured at 640 nm after 2h.

The optical density at 640 nm was measured using SpectraMax Plus 384 Microplate Reader. A negative trend was found as a higher amount of lipofectamine yielded lower absorbance. Hence, the lowest amount of transfection reagent, 1 µl was used for stimulation experiments.

4.7 QuantiBlue assay of mutated *CSF3R*

G-CSF was used to induce STAT3 signaling through the WT or mutated G-CSF receptor and IL-6 was used as a positive control in untransfected cells. *CSF3R* WT and the mutations p.(Gly27Arg) and p.(Thr640Asn), as well as negative and positive controls, were investigated with the assay (Figure 4.10). Background signaling from unstimulated cells were removed and the results normalized to the WT signal. The p.(Thr640Asn) mutation showed a slightly reduced signal compared to the WT cells. The p.(Gly27Arg) mutation had a dramatic effect on STAT3 signaling and could not induce any detectable signal higher than the unstimulated control cells.

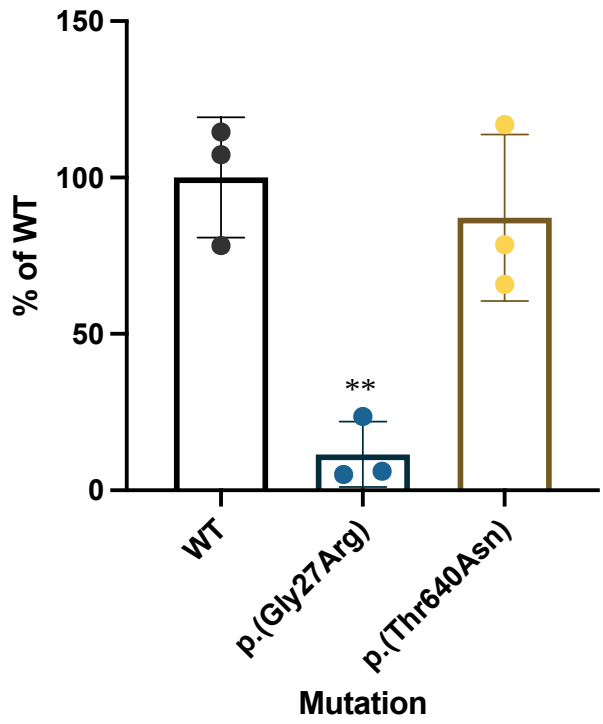

Figure 4.10 – STAT3 signaling through the G-CSF receptor when stimulated with G-CSF. Two mutations and the WT *CSF3R* were transfected into IL-6 reporter cells. The cells were either unstimulated or stimulated with G-CSF or IL-6. Absorbance was measured at 640 nm after 2 h, background signal was removed and the % of WT signaling was calculated.

*: P-value ≤ 0.05; **: P-value ≤ 0.01; ***: P-value ≤ 0.001.

5. Discussion

Signaling through the G-CSF receptor is responsible for the production and maturation of neutrophil granulocytes. The receptor is crucial for differentiation and proliferation of cells throughout granulopoiesis. Mutations in the receptor can have deactivating effects leading to the disorder SCN7. Gain of function mutations can also occur adding risk of getting chronic neutrophilic leukemia (CNL) [45]. SCN patients with inactivating pathogenic mutations in the *CSF3R* gene are given the diagnosis of SCN7, but this process is not always straight forward. It can be challenging to determine whether a novel missense mutation in the *CSF3R* gene is benign or pathogenic. Functional assays for mutations in the *CSF3R* gene would therefore be very beneficial for clinicians when diagnosing patients with suspected CNL or SCN. The treatment of SCN also differs based on the underlying cause of the disease [95, 96]. G-CSF is given as general treatment for SCN, but G-CSF will not increase total neutrophil count in the presence of a pathogenic loss-of-function *CSF3R*-mutation. A test to aid in deciding the effects of novel *CSF3R* mutations would also contribute to giving the right medication for SCN patients as early as possible. Our aim was therefore to establish functional tests where the effects of missense *CSF3R* mutations on signaling could be detected and characterized.

Phenotyping with antibodies for the G-CSF receptor revealed the presence of the receptor on both monocytes and granulocytes. We tried to stimulate PBL with G-CSF to study signaling protein activation in granulocytes but did not see much downstream signaling. We did however see a considerable increase in monocyte signaling protein activation following G-CSF stimulation in PBMC, indicating receptor activation. In particular, phospho-signals from STAT3 and STAT5 increased significantly after G-CSF stimulation in monocytes. A colorimetric reporter-assay was established to study STAT3-signaling induced by WT and mutated G-CSF receptors. The assay was able to detect and quantify STAT3 signaling upon G-CSF stimulation and was used to reclassify the *CSF3R* mutation p.(Gly27Arg) from VUS to likely pathogenic.

5.1 Granulocytes in isolated PBL showed little response to stimulation with G-CSF in vitro

Six different concentrations of G-CSF were used as they represented the range of concentrations mostly used in the literature, 0.316 - 100 ng/ml. We observed little change in phospho-signal from STAT3, STAT5, Akt and Erk1/2 in granulocytes stimulated by adding

G-CSF to whole blood. This may indicate that G-CSF stimulation has little to no effect on mature granulocytes, at least in our experimental setup. The activation of these four proteins through G-CSF stimulation has been known for many years. Tian and colleagues showed the G-CSF-activation of STAT3 and STAT5 as early as in 1996 [97]. The effect of G-CSF on stem cells are clear, as it initiates their release from the BM into circulation [98]. G-CSF is also well known for initiating the development of HSC into mature granulocytes [99]. The response of G-CSF stimulation in mature granulocytes are still debated, but there are studies indicating that the stimulation indeed induces activation of neutrophils [100-102]. These studies vary from our experiments in multiple ways; **1)** They were done on isolated neutrophils and not in PBL as done here. **2)** There are methodological differences as none of the studies have used flow cytometry. **3)** The cells used have been cultured in human serum. The findings indicate that G-CSF alone cannot activate mature neutrophils but rather primes them for activation by other signaling molecules. These studies have not assessed kinase signaling following G-CSF stimulation, as we have, but rather activation of neutrophils in terms of different phenotypic characteristics. The main method for studying G-CSF signaling have been through western blot done on secondary cell-lines, but a study by Redell and colleagues used phospho-flow on BM cells and found that around half of the cells activated either STAT3 or STAT5 signaling pathway [103]. However, many studies such as the one from Everson and colleagues show that it is possible to detect changes in phospho-signal in isolated PBL [104]. Still, our experiments found little indication of downstream signaling activity in the mature granulocytes. Therefore, it is reasonable to believe that blood granulocytes are not able to activate classical G-CSF signaling upon stimulation with G-CSF. It may also be that an agent in the blood quenches G-CSF.

As we could not detect any activation of the signaling proteins by the G-CSF receptor in PBL, we could have used bone marrow cells, as we know that those cells respond to G-CSF stimulation. Many studies done on G-CSF stimulation are done on bone marrow cells [99]. G-CSF receptor amount increases as stem cells differentiate towards mature granulocytes [105, 106]. It has been shown that only a small number of receptors needs to be activated for G-CSF to reach half of its maximal activity [106]. Therefore, BM cells could be used even though they have fewer receptors present than matured neutrophils. However, it is more challenging to acquire human BM cells compared to blood. Sampling of BM can be painful and thus requires ethical consideration. The difficulties around acquisition of BM cells would make the assay hard to carry out in a rapid manner for these patients.

5.2 Methanol permeabilization resulted in increased signal from surface markers CD66b and CD89

Permeabilization with chemicals such as methanol is necessary for phospho-specific antibodies to enter the cells, but methanol can degrade important surface markers [107]. A difference was shown between the different surface markers as anti-CD16 showed no difference in permeabilized and non-permeabilized cells. This result match the results by Lazarus and colleagues who looked at the impact of permeabilization on different surface markers [108]. The result indicates that dissolving of lipids in the membrane does not affect CD16. This may be because the ligand-binding domain of the receptor is located entirely on the outside of the membrane. Permeabilization has been found to positively affect CD66b detection [109]. The same results are found for both CD66b and CD89 in the current study. The permeabilization may make CD66b and CD89 more available for their antibodies. The differences found between CD16 and CD89/CD66b are most likely due to differences in the receptor composition and placement in the membrane.

5.3 The effect of G-CSF stimulation on monocytes from isolated PBMC

Due to the lack of signaling in stimulated granulocytes we wanted to examine the signaling patterns of G-CSF receptor in monocytes and therefore decided to isolate PBMC as a source of these cells. The G-CSF receptor is, as shown earlier, present on the surface of monocytes. Phospho-signaling through the G-CSF receptor of monocytes has not been described in the literature yet. However, phospho-flow cytometry has been done on monocytes with success [110]. We saw a marked improvement in phospho-signal response when studying the monocytes in comparison to the granulocytes, and the G-CSF stimulation induced a significant increase in signaling for STAT3 and STAT5. This is in line with experiments done by Saito and colleagues, and later Christopher and colleagues who found that G-CSF stimulation of monocytes activates downstream effects, such as cytokine secretion and stem cell mobilization [111, 112]. The current study is however the first study quantifying this phospho-signal by phospho-flow cytometry.

Some of our data suggest that the increase of G-CSF concentration in circulation is in correlation with higher signaling protein activity, as the five-fold increase in G-CSF concentration resulted in higher signal from the phospho-antibodies. This was true for STAT5 which showed a much higher fold change in the 100 ng/ml sample. STAT3 and Akt did not

show the same results where the 100 ng/ml sample only showed a small increase in fold-change compared to the 20 ng/ml sample. This trend has been shown clinically, where an increase in the amount of G-CSF administrated has led to increased mobilization of MP cells [113]. The concentrations of G-CSF chosen in the current study, 20 ng/ml and 100 ng/ml are highly physiological relevant as they cover the recommended dosage of G-CSF for a patient at 70 kg, 50 – 87.5 ng/ml [95]. Sepsis patients are treated with a significantly higher dosage at 300 μg/day [114] Higher dosages are recommended for severe neutropenia patients. Adding higher doses in our experiments could perhaps have shown a dose-response in kinase activation even clearer.

Although phospho-flow cytometry of PBMC have not yet been done with G-CSF stimulation by others; it has however been done after GM-CSF stimulation. Lee and colleagues studied the signal from different STATs following PBMC stimulation with GM-CSF [115]. Their findings are in line with ours, in that they show a detectable phospho-signal in PBMC after stimulation with a CSF. This further strengthens phospo-flow as a method to study changes in CSF-receptors.

The CSF-family of growth factors is diverse, as explained in Table **1.1** of section 1.3.1. The factors are responsible for stimulating the production of blood cells, what blood cells are produced from which stimulant overlap slightly, as granulocytes can be produced from both G-CSF and GM-CSF signaling and macrophages from both GM-CSF and M-CSF [100, 116]. G-CSF will as previously stated be prescribed to patients with too few neutrophils in circulation, and GM-CSF will be prescribed to SCN7 patients. The potential of crosstalk in the current PBMC experiment cannot be ruled out as these factors share a common function. The signaling pathways of the CSF-family is also very similar, all the three growth factors signal through both the PI3K and Akt pathways [117]. G-CSF and GM-CSF also both signal through the STAT5 signaling pathway, leading to similar transcriptional change. Indeed, some crosstalk between the growth factors have been reported as GM-CSF signaling leads to an overexpression of M-CSF in monocytes [118]. Interactions between several CSFs in mouse have been found to increase the number of cells produced by the BM compared to with only a single CSF [119]. The cells produced were also larger in size when cultures were stimulated with G-CSF in combination with either M-CSF or GM-CSF. This may indicate that there really is some crosstalk in the downstream signaling from the CSFs. Castellani and colleagues found evidence of crosstalk between G-CSF and GM-CSF [120]. Stimulation with

G-CSF or GM-CSF individually showed very similar effects on production of ROS. A priming effect have also been suggested, where effects of GM-CSF are enhanced after a treatment with G-CSF [121]. A study by Park and colleagues looked at competitive binding between G-CSF, GM-CSF and IL-3 (previously known as multi-CSF) [122]. They found that IL-3 binding to its receptor were inhibited by G-CSF and GM-CSF, and that GM-CSF binding was inhibited by IL-3 and G-CSF.

Our work on signaling following G-CSF stimulation did not account for any potential crosstalk between the different colony stimulating factors. Any changes in protein signaling discussed in this study needs to be viewed while keeping crosstalk in mind, as there is a potential of changes in signaling activity via other pathways.

The lymphocytes used as positive controls for the study all displayed the expected activity induced by the relevant stimulant. This indicates that the stimulations and antibody staining worked as intended. G-CSF stimulation of the lymphocytes did show a small STAT5 signal increase. This is unexpected as G-CSF stimulation should not affect the lymphocytes as they do not have the G-CSF receptor. One potential reason may be that the gate for the lymphocytes also contain some other cells that may possess the G-CSF receptor, e.g., DCs. It may also be that high dosage of G-CSF can stimulate other receptors leading to STAT5 activation.

Overall, the phospho-flow cytometry of monocytes from isolated PBMC did show great potential to be used as a basis for a functional assay for assessing mutations found in the *CSF3R* gene. It is worth mentioning that only three donors were tested, and that there are some variations in the data collected from the different donors. Despite this, we successfully demonstrated increased signaling when stimulated with G-CSF.

5.3.1 Phospho-flow cytometry as a method to assess the effects of unknown mutations

The flow cytometric assay established in this study was created as a way of assessing signaling effects of mutations in the *CSF3R* gene. Phospho-flow works well for identifying downstream signaling in blood samples collected from donors. Quantification of signal seems to be a bit more challenging, with background signal as one of many potential interferences. We still see the potential of such an assay in the help of diagnosing SCN7 patients, where a semi-quantitative measure of signaling activity in many cases will be sufficient. The assay

therefore seems applicable for assessing signaling effects in patients carrying variants of unknown significance in the G-CSF receptor gene, and similar techniques could be applicable for other genes as well. Most of the work done on characterizing *CSF3R* variants are done in silico, so this assay could be of great value in functionally testing the effect of these variants in vitro.

5.4 *CSF3R* mutations alter STAT3 signaling in HEK-Blue cells

A reporter-assay for quantifying STAT3 signaling following G-CSF stimulation was created by transfecting a commercially available IL-6 cell line with plasmids encoding WT and mutant versions of *CSF3R*.

Some difficulties were encountered during transformation and purification of the plasmid, pUNO-CSF3Ra (Figure 7.1 in appendix). A very high concentration of blasticidin, the selection marker, was needed for plasmid selection. Both the information of what bacterial strains blasticidin interact with and the bacterial strains available was limited. A plasmid with a different selection marker would have been preferred had the project been repeated. Still, we were able to establish the reporter assay for WT *CSF3R* and we were able to test the function of the clinically interesting variant p.(Gly27Arg).

No functional testing has been done previously on the p.(Gly27Arg) mutation and molecular interpretation using standardized guidelines classifies it as a VUS [123]. Using our developed reporter assay, STAT3 activity was found to be barely detectable. Our results therefore suggest that the p.(Gly27Arg) mutation has a large impact on STAT3 signaling following G-CSF stimulation of the receptor. The mutation is present in the N-terminal part of the protein, and we hypothesize that the non-conservative mutation from a small, non-polar glycine to a large and positively charged arginine changes the structure of the protein leading to a reduced affinity to the ligand. This may explain the chronic infections experienced from patients with the mutation, as lowered signaling will lead to fewer neutrophils in circulation. Very few *CSF3R* mutations have been described in the N-terminus of G-CSF receptor, but the mutation p.(Leu89Pro) was observed in a case of SCN in Turkey [124]. This mutation is both non-conservative and present at the IG-like C2 domain. The patient was compound heterozygous with another *CSF3R* mutation, p.(Arg190His), but did still respond to treatment with G-CSF. This indicates that at least one of the two mutations negatively impacted G-CSF receptor

function, but also that some residual activity remained. The amino acids Phe75, Gln87 and Gln91 has been found to form a binding site for G-CSF [125]. Non-conservative mutations in this area have the potential to alter ligand binding affinity, which could lead to decreased receptor signaling. A study by Bitard and colleagues looked at the effects of mutations in Ig-like domains of gp130 [126]. They found that mutations in this domain have great impacts of ligand binding affinity. Small missense mutations had the potential to increase and decrease binding affinity. G-CSF receptor has a very similar structure to gp130 with five fibronectin type 3 domains and an Ig-like C2 domain. This supports our claims that the p.(Gly27Arg) mutation on the Ig-like domain of the G-CSF receptor lowers the ligands affinity to the receptor.

Only one test was conducted to characterize the p.(Gly27Arg) mutation, but our results match the clinical history of the family described in section 1.5.6. Being a heterozygous carrier for the mutation and the WT gene does not result in onset of SCN, but the mutation leads to neutropenia when it is present with another known pathogenic mutation (such as p.(Trp547Ter). It needs to be stated that this one test does not tell us the entire story of the functionality of the mutation but gives us indications of deficient receptor activity. It would be interesting to test patient-blood on our established phospho-flow assay to investigate the findings of the mutagenesis-assay further. This would be beneficial for characterization of the other signaling pathways than STAT3 and it would indicate whether the reporter-assay worked as intended.

The second variant tested; p.(Thr640Asn) has been found to be oncogenic [90]. The study by Maxson and colleagues did find an increased JAK signaling via upregulation of STAT3 protein in a western blot. STAT3 signaling in our study was slightly decreased with the p.(Thr640Asn) mutation compared to the WT. This may indicate some flaws in the assay we created and means that more work and optimalization needs to be done to quantify the STAT3 signaling, in particular for gain-of-function mutations such as p.(Thr640Asn). STAT3 might not be upregulated the same way in our HEK-cells as it is in hematopoietic cells. An example would be upstream regulatory elements which might not be present in the HEK cells. Activating mutations can be very different to inactivating mutations when it comes to kinetics and incubation time. Our test was ultimately conducted to check the signaling properties of the p.(Gly27Arg) mutation, and similar loss-of-function or hypomorphic mutations. The

levels of signaling from the p.(Thr640Asn) mutation were very similar to that of the WT, with one of the replicates having higher signal than any of the WT replicates.
The assay did however manage to detect the presence of STAT3 signaling after stimulation of the WT G-CSFR and indicated that the p.(Gly27Arg) mutation has a severe impact on the STAT3 signaling following G-CSF stimulation. The assay therefore appears promising as a tool to assess if novel mutations in the *CSF3R* gene identified in the future leads to inactivation of signaling through STAT3. Absence of STAT3 signaling indicates changes in overall receptor signaling. Our focus for this test has been SCN7 and inactivating mutations, which this assay indeed apparently manages to pick up. With more optimization the assay could potentially be used for activating leukemia-associated mutations as well.

5.5 Limitations of the study

The study had some limitations. Optimalization of the flow assay was time consuming and this limited the number of controls we could run in our project. This did also lead to a reduction of the statistical power of our analysis. Two different flow cytometers were also used during the project, which meant that some of the optimalization done on one cytometer needed to be done all over again on the new cytometer.

Running SCN7 patient blood through our flow assay would be optimal but samples of blood from individuals with SCN7 were not available during the work with this master's thesis. This means that the assay was only tested with healthy donors The assay should still be able to detect reduced signaling, but a confirmation by running patient blood would have been optimal and should be carried out in the near future.

Cell loss was a problem throughout our work. It may have been a result of a lot of washing steps when staining with antibodies [127]. The amount of PBMC and consequently monocytes in the donor blood did also vary from donor to donor. Monocyte number are known to be between 5 and 10 % of all leukocytes [15]. The amount of blood from each donor received from the blood bank limits the number of monocytes that can be isolated from each donor. There might not be enough monocytes left to perform meaningful experiments after cell- loss and -death are taken into consideration.

The QuantiBlue assay did also have some limitations. It is common practice to use an empty vector as control plasmid when performing a transfection. This was not done when transfecting the cells with the mutated receptor. Instead, WT receptor was used as a positive control, and signaling through the receptor did control that the transfection was successful. The assay did also have quite varying values from run to run. The light absorption of the assay did quickly change from low to very high values when the color reagent was added. This meant that it was difficult to measure the change of absorption more dynamically over time, which could have been for quantifying changes induced by activating mutations.

5.6 Conclusion

In summary, we have created two different assays for assessing signaling through the G-CSF receptor following G-CSF stimulation. The first assay is a flow assay that analyses phospho-signals in monocytes isolated from peripheral blood samples. Monocytes carry the G-CSF receptor and stimulation with G-CSF will lead to an increase in downstream activities of several signaling proteins. The assay can detect changes in this signaling activity by an increase in phosphorylation of STAT3, STAT5, Erk1/2 and Akt. Tests done on healthy donor blood reveal a significant increase in phosphorylation of STAT3 and STAT5 when the monocytes are stimulated with G-CSF. This property can be used to examine *CSF3R* mutations in patient blood.

A second assay was established, this one to edit and assess functionality of missense *CSF3R* mutations on STAT3 signaling. Two mutations, one activating and one VUS were tested with the assay. The activating variant, p.(Thr640Asn) did show similar STAT3 activity to the WT. This may indicate that the assay is more useful at detecting a STAT3 signal compared to quantifying the amount of signaling. Further work needs to be done to optimize the assay for activating mutations. However, testing the VUS p.(Gly27Arg), detected in patients with SCN7, showed that STAT3 signaling was almost negligible when compared to the WT, thus indicating that the variant may be pathogenic.

The STAT3-assay did also show varying values from run to run and more testing needs to be done to know if the results from the assay are reliable. No known inactivating mutations was run through the study as some difficulties were had for the mutagenesis and bacterial transformations. We have shown indications that the p.(Gly27Arg) mutation is pathogenic or benign, but further work needs to be done to draw further conclusion.

5.7 Future perspectives

As previously stated, our phospho-flow assay was only run on healthy control PBMC. Running the assay with blood from SCN7 patients would be beneficial in confirming our findings. This would give us information on whether the increased phospho-signal we found was because of G-CSF signaling or if there are other mechanisms such as CSF-cross talk that increase signaling protein activation. This could also be studied with different stimulation experiments with combinations of G-CSF, M-CSF and GM-CSF. The HEK cells would be transfected with M-CSF or GM-CSF receptor in combination with G-CSF receptor and stimulated with G-CSF. STAT3 signaling values would be measured using the reporter assay and compared between the different combinations of receptors. Increases in STAT3 signaling would indicate cross talk were G-CSF may have bound to either of the two other receptors.

It would also be interesting to run more healthy donors through the phospho-flow assay to see if it was possible to detect significant changes from Erk1/2 and Akt as found for STAT3 and STAT5. These findings would also tell us if maybe only some individuals activate Erk1/2 and Akt after G-CSF stimulation as we a high variance in signal from the two kinases.

Despite numerous attempts, mutagenesis of p.(Trp574Ter) was unsuccessful. Further work would include testing different mutagenesis primers to create the same amino acid change but with a different nucleotide change. This would open up for studying the effects of both p.(Gly27Arg) and p.(Trp547Ter) together.

In our study, multiple antibodies have been used in flow cytometry. The specificity of these antibodies could be shown by western blot analysis. The phospho-antibodies would be particularly interesting to validate the specificity of, as these are an intricate part of the phospho-flow assay.

The reporter assay was established around inactivating mutations and work could be done to optimize it for activating mutations. It would also be interesting to investigate regulatory elements of *CSF3R* in vivo in comparison to in pUNO-hCSF3Ra.

6. References

1. Parkin, J. and B. Cohen, *An overview of the immune system.* Lancet, 2001. **357**(9270): p. 1777-89.
2. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
3. Hato, T. and P.C. Dagher, *How the Innate Immune System Senses Trouble and Causes Trouble.* Clin J Am Soc Nephrol, 2015. **10**(8): p. 1459-69.
4. Delves, P.J. and I.M. Roitt, *The immune system. First of two parts.* N Engl J Med, 2000. **343**(1): p. 37-49.
5. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nat Rev Immunol, 2020. **20**(6): p. 375-388.
6. Riera Romo, M., D. Perez-Martinez, and C. Castillo Ferrer, *Innate immunity in vertebrates: an overview.* Immunology, 2016. **148**(2): p. 125-39.
7. Niyonsaba, F., et al., *Multifunctional antimicrobial proteins and peptides: natural activators of immune systems.* Curr Pharm Des, 2009. **15**(21): p. 2393-413.
8. Haniffa, M., M. Gunawan, and L. Jardine, *Human skin dendritic cells in health and disease.* J Dermatol Sci, 2015. **77**(2): p. 85-92.
9. Netea, M.G. and J.W. van der Meer, *Immunodeficiency and genetic defects of pattern-recognition receptors.* N Engl J Med, 2011. **364**(1): p. 60-70.
10. Janeway, C.A., Jr., *Approaching the asymptote? Evolution and revolution in immunology.* Cold Spring Harb Symp Quant Biol, 1989. **54 Pt 1**: p. 1-13.
11. Medzhitov, R., *Recognition of microorganisms and activation of the immune response.* Nature, 2007. **449**(7164): p. 819-26.
12. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.
13. Kumar, H., T. Kawai, and S. Akira, *Toll-like receptors and innate immunity.* Biochem Biophys Res Commun, 2009. **388**(4): p. 621-5.
14. Lee, W.L., R.E. Harrison, and S. Grinstein, *Phagocytosis by neutrophils.* Microbes Infect, 2003. **5**(14): p. 1299-306.
15. Prinyakupt, J. and C. Pluempitiwiriyawej, *Segmentation of white blood cells and comparison of cell morphology by linear and naive Bayes classifiers.* Biomed Eng Online, 2015. **14**: p. 63.
16. Savina, A. and S. Amigorena, *Phagocytosis and antigen presentation in dendritic cells.* Immunol Rev, 2007. **219**: p. 143-56.
17. Roche, P.A. and K. Furuta, *The ins and outs of MHC class II-mediated antigen processing and presentation.* Nat Rev Immunol, 2015. **15**(4): p. 203-16.
18. Ramirez, G.A., et al., *Eosinophils from Physiology to Disease: A Comprehensive Review.* Biomed Res Int, 2018. **2018**: p. 9095275.
19. Siracusa, M.C., et al., *Basophils and allergic inflammation.* J Allergy Clin Immunol, 2013. **132**(4): p. 789-801; quiz 788.
20. Rosales, C., *Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?* Front Physiol, 2018. **9**: p. 113.
21. Entschladen, F., et al., *T lymphocytes and neutrophil granulocytes differ in regulatory signaling and migratory dynamics with regard to spontaneous locomotion and chemotaxis.* Cell Immunol, 2000. **199**(2): p. 104-14.
22. Hager, M., J.B. Cowland, and N. Borregaard, *Neutrophil granules in health and disease.* J Intern Med, 2010. **268**(1): p. 25-34.

23. Pillay, J., et al., *A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1.* J Clin Invest, 2012. **122**(1): p. 327-36.
24. Winterbourn, C.C., A.J. Kettle, and M.B. Hampton, *Reactive Oxygen Species and Neutrophil Function.* Annu Rev Biochem, 2016. **85**: p. 765-92.
25. Nguyen, G.T., E.R. Green, and J. Mecsas, *Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance.* Front Cell Infect Microbiol, 2017. **7**: p. 373.
26. Lacy, P., *Mechanisms of degranulation in neutrophils.* Allergy Asthma Clin Immunol, 2006. **2**(3): p. 98-108.
27. Papayannopoulos, V., *Neutrophil extracellular traps in immunity and disease.* Nat Rev Immunol, 2018. **18**(2): p. 134-147.
28. Teng, T.S., et al., *Neutrophils and Immunity: From Bactericidal Action to Being Conquered.* J Immunol Res, 2017. **2017**: p. 9671604.
29. Schultze, J.L., E. Mass, and A. Schlitzer, *Emerging Principles in Myelopoiesis at Homeostasis and during Infection and Inflammation.* Immunity, 2019. **50**(2): p. 288-301.
30. Zhang, J.M. and J. An, *Cytokines, inflammation, and pain.* Int Anesthesiol Clin, 2007. **45**(2): p. 27-37.
31. Dancey, J.T., et al., *Neutrophil kinetics in man.* J Clin Invest, 1976. **58**(3): p. 705-15.
32. Ng, A.P. and W.S. Alexander, *Haematopoietic stem cells: past, present and future.* Cell Death Discov, 2017. **3**: p. 17002.
33. He, S., D. Nakada, and S.J. Morrison, *Mechanisms of stem cell self-renewal.* Annu Rev Cell Dev Biol, 2009. **25**: p. 377-406.
34. Weiskopf, K., et al., *Myeloid Cell Origins, Differentiation, and Clinical Implications.* Microbiol Spectr, 2016. **4**(5).
35. Manz, M.G. and S. Boettcher, *Emergency granulopoiesis.* Nat Rev Immunol, 2014. **14**(5): p. 302-14.
36. Mehta, H.M. and S.J. Corey, *G-CSF, the guardian of granulopoiesis.* Semin Immunol, 2021. **54**: p. 101515.
37. Hidalgo, A., et al., *The Neutrophil Life Cycle.* Trends Immunol, 2019. **40**(7): p. 584-597.
38. Martin, K.R., et al., *G-CSF - A double edge sword in neutrophil mediated immunity.* Semin Immunol, 2021. **54**: p. 101516.
39. Gregory, A.D., et al., *Regulation of systemic and local neutrophil responses by G-CSF during pulmonary Pseudomonas aeruginosa infection.* Blood, 2007. **109**(8): p. 3235-43.
40. Cebon, J., et al., *Endogenous haemopoietic growth factors in neutropenia and infection.* Br J Haematol, 1994. **86**(2): p. 265-74.
41. Panopoulos, A.D. and S.S. Watowich, *Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis.* Cytokine, 2008. **42**(3): p. 277-88.
42. Kanegane, H., et al., *Flow cytometry-based diagnosis of primary immunodeficiency diseases.* Allergol Int, 2018. **67**(1): p. 43-54.
43. Petit, I., et al., *G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4.* Nat Immunol, 2002. **3**(7): p. 687-94.
44. Fukunaga, R., et al., *Functional domains of the granulocyte colony-stimulating factor receptor.* EMBO J, 1991. **10**(10): p. 2855-65.
45. Dwivedi, P. and K.D. Greis, *Granulocyte colony-stimulating factor receptor signaling in severe congenital neutropenia, chronic neutrophilic leukemia, and related malignancies.* Exp Hematol, 2017. **46**: p. 9-20.

46. Barge, R.M., et al., *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): p. 2148-53.
47. de Koning, J.P., et al., *Specific involvement of tyrosine 764 of human granulocyte colony-stimulating factor receptor in signal transduction mediated by p145/Shc/GRB2 or p90/GRB2 complexes.* Blood, 1996. **87**(1): p. 132-40.
48. Dagil, R., et al., *The WSXWS motif in cytokine receptors is a molecular switch involved in receptor activation: insight from structures of the prolactin receptor.* Structure, 2012. **20**(2): p. 270-82.
49. Hilton, D.J., et al., *Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor.* Journal of Biological Chemistry, 1996. **271**(9): p. 4699-4708.
50. Roskoski, R., Jr., *A historical overview of protein kinases and their targeted small molecule inhibitors.* Pharmacol Res, 2015. **100**: p. 1-23.
51. Park, S.D., et al., *A review of granulocyte colony-stimulating factor receptor signaling and regulation with implications for cancer.* Front Oncol, 2022. **12**: p. 932608.
52. Dong, F., et al., *Stimulation of Stat5 by granulocyte colony-stimulating factor (G-CSF) is modulated by two distinct cytoplasmic regions of the G-CSF receptor.* J Immunol, 1998. **161**(12): p. 6503-9.
53. Stefan, E. and K. Bister, *MYC and RAF: Key Effectors in Cellular Signaling and Major Drivers in Human Cancer.* Curr Top Microbiol Immunol, 2017. **407**: p. 117-151.
54. Hemmings, B.A. and D.F. Restuccia, *PI3K-PKB/Akt pathway.* Cold Spring Harb Perspect Biol, 2012. **4**(9): p. a011189.
55. Liu, F., J. Poursine-Laurent, and D.C. Link, *Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF.* Blood, 2000. **95**(10): p. 3025-31.
56. Wong, D. and K. Dorovini-Zis, *Expression of vascular cell adhesion molecule-1 (VCAM-1) by human brain microvessel endothelial cells in primary culture.* Microvasc Res, 1995. **49**(3): p. 325-39.
57. D'Apuzzo, M., et al., *The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4.* Eur J Immunol, 1997. **27**(7): p. 1788-93.
58. Levesque, J.P., et al., *Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor.* Blood, 2001. **98**(5): p. 1289-97.
59. Stoikou, M., et al., *G-CSF Infusion for Stem Cell Mobilization Transiently Increases Serum Cell-Free DNA and Protease Concentrations.* Front Med (Lausanne), 2020. **7**: p. 155.
60. Wadhams, G.H. and J.P. Armitage, *Making sense of it all: Bacterial chemotaxis.* Nature Reviews Molecular Cell Biology, 2004. **5**(12): p. 1024-1037.
61. Petri, B. and M.J. Sanz, *Neutrophil chemotaxis.* Cell and Tissue Research, 2018. **371**(3): p. 425-436.
62. Yong, K.L., *Granulocyte colony-stimulating factor (G-CSF) increases neutrophil migration across vascular endothelium independent of an effect on adhesion: comparison with granulocyte-macrophage colony-stimulating factor (GM-CSF).* Br J Haematol, 1996. **94**(1): p. 40-7.

63. Molteni, M., S. Gemma, and C. Rossetti, *The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation.* Mediators Inflamm, 2016. **2016**: p. 6978936.
64. Sugimoto, M.A., et al., *Resolution of Inflammation: What Controls Its Onset?* Front Immunol, 2016. **7**: p. 160.
65. Fox, S., et al., *Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease.* J Innate Immun, 2010. **2**(3): p. 216-27.
66. Stark, M.A., et al., *Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17.* Immunity, 2005. **22**(3): p. 285-94.
67. Greenlee-Wacker, M.C., *Clearance of apoptotic neutrophils and resolution of inflammation.* Immunol Rev, 2016. **273**(1): p. 357-70.
68. Szepanowski, F., et al., *Secondary Immunodeficiency and Risk of Infection Following Immune Therapies in Neurology.* CNS Drugs, 2021. **35**(11): p. 1173-1188.
69. Buckley, R.H., *Primary immunodeficiency diseases due to defects in lymphocytes.* N Engl J Med, 2000. **343**(18): p. 1313-24.
70. Lekstrom-Himes, J.A. and J.I. Gallin, *Immunodeficiency diseases caused by defects in phagocytes.* N Engl J Med, 2000. **343**(23): p. 1703-14.
71. Sarma, J.V. and P.A. Ward, *The complement system.* Cell Tissue Res, 2011. **343**(1): p. 227-35.
72. Smith, T. and C. Cunningham-Rundles, *Primary B-cell immunodeficiencies.* Hum Immunol, 2019. **80**(6): p. 351-362.
73. Bos, M.M., et al., *Bloodstream infections in patients with or without cancer in a large community hospital.* Infection, 2013. **41**(5): p. 949-58.
74. Netea, M.G., F.L. van de Veerdonk, and J.W. van der Meer, *Primary immunodeficiencies of pattern recognition receptors.* J Intern Med, 2012. **272**(6): p. 517-27.
75. Linton, S.M. and B.P. Morgan, *Properdin deficiency and meningococcal disease--identifying those most at risk.* Clin Exp Immunol, 1999. **118**(2): p. 189-91.
76. Leszcynska, M., et al., *Brain Abscess as Severe Presentation of Specific Granule Deficiency.* Front Pediatr, 2020. **8**: p. 117.
77. Skokowa, J., et al., *Severe congenital neutropenias.* Nat Rev Dis Primers, 2017. **3**: p. 17032.
78. Tahir, N. and F. Zahra, *Neutrophilia*, in *StatPearls*. 2023: Treasure Island (FL).
79. Boxer, L.A., *How to approach neutropenia.* Hematology Am Soc Hematol Educ Program, 2012. **2012**: p. 174-82.
80. Klein, C., *Genetic Defects in Severe Congenital Neutropenia: Emerging Insights into Life and Death of Human Neutrophil Granulocytes.* Annual Review of Immunology, Vol 29, 2011. **29**: p. 399-413.
81. Triot, A., et al., *Inherited biallelic CSF3R mutations in severe congenital neutropenia.* Blood, 2014. **123**(24): p. 3811-3817.
82. Metcalf, D., *Hematopoietic cytokines.* Blood, 2008. **111**(2): p. 485-91.
83. Eyles, J.L., et al., *A key role for G-CSF-induced neutrophil production and trafficking during inflammatory arthritis.* Blood, 2008. **112**(13): p. 5193-201.
84. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
85. Chakraborty, A., et al., *Granulocyte colony-stimulating factor promotes adhesion of neutrophils.* Am J Physiol Cell Physiol, 2003. **284**(1): p. C103-10.
86. Fullerton, J.N. and D.W. Gilroy, *Resolution of inflammation: a new therapeutic frontier.* Nat Rev Drug Discov, 2016. **15**(8): p. 551-67.

87. Chavez-Galan, L., et al., *Much More than M1 and M2 Macrophages, There are also CD169(+) and TCR(+) Macrophages.* Front Immunol, 2015. **6**: p. 263.
88. Kwon, T., I.B. Lamster, and L. Levin, *Current Concepts in the Management of Periodontitis.* Int Dent J, 2021. **71**(6): p. 462-476.
89. Zaromb, A., et al., *Periodontitis as a manifestation of chronic benign neutropenia.* J Periodontol, 2006. **77**(11): p. 1921-6.
90. Maxson, J.E., et al., *The Colony-Stimulating Factor 3 Receptor T640N Mutation Is Oncogenic, Sensitive to JAK Inhibition, and Mimics T618I.* Clin Cancer Res, 2016. **22**(3): p. 757-64.
91. Plo, I., et al., *An activating mutation in the CSF3R gene induces a hereditary chronic neutrophilia.* J Exp Med, 2009. **206**(8): p. 1701-7.
92. Maxson, J.E., et al., *Ligand independence of the T618I mutation in the colony-stimulating factor 3 receptor (CSF3R) protein results from loss of O-linked glycosylation and increased receptor dimerization.* J Biol Chem, 2014. **289**(9): p. 5820-7.
93. Klimiankou, M., et al., *GM-CSF stimulates granulopoiesis in a congenital neutropenia patient with loss-of-function biallelic heterozygous CSF3R mutations.* Blood, 2015. **126**(15): p. 1865-7.
94. Liongue, C. and A.C. Ward, *Granulocyte colony-stimulating factor receptor mutations in myeloid malignancy.* Front Oncol, 2014. **4**: p. 93.
95. Mehta, H.M., M. Malandra, and S.J. Corey, *G-CSF and GM-CSF in Neutropenia.* J Immunol, 2015. **195**(4): p. 1341-9.
96. Dale, D.C. and A.A. Bolyard, *An update on the diagnosis and treatment of chronic idiopathic neutropenia.* Curr Opin Hematol, 2017. **24**(1): p. 46-53.
97. Tian, S.S., et al., *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): p. 4435-44.
98. Tamura, M., et al., *Induction of neutrophilic granulocytosis in mice by administration of purified human native granulocyte colony-stimulating factor (G-CSF).* Biochem Biophys Res Commun, 1987. **142**(2): p. 454-60.
99. Bradley, T.R. and D. Metcalf, *The growth of mouse bone marrow cells in vitro.* Aust J Exp Biol Med Sci, 1966. **44**(3): p. 287-99.
100. Metcalf, D., *The colony-stimulating factors and cancer.* Nat Rev Cancer, 2010. **10**(6): p. 425-34.
101. Roilides, E., et al., *Granulocyte colony-stimulating factor enhances the phagocytic and bactericidal activity of normal and defective human neutrophils.* J Infect Dis, 1991. **163**(3): p. 579-83.
102. Demetri, G.D. and J.D. Griffin, *Granulocyte Colony-Stimulating Factor and Its Receptor.* Blood, 1991. **78**(11): p. 2791-2808.
103. Redell, M.S., et al., *FACS analysis of Stat3/5 signaling reveals sensitivity to G-CSF and IL-6 as a significant prognostic factor in pediatric AML: a Children's Oncology Group report.* Blood, 2013. **121**(7): p. 1083-93.
104. Everson, R.G., et al., *Cytokine responsiveness of CD8(+) T cells is a reproducible biomarker for the clinical efficacy of dendritic cell vaccination in glioblastoma patients.* J Immunother Cancer, 2014. **2**: p. 10.
105. Nicola, N.A. and D. Metcalf, *Binding of the differentiation-inducer, granulocyte-colony-stimulating factor, to responsive but not unresponsive leukemic cell lines.* Proc Natl Acad Sci U S A, 1984. **81**(12): p. 3765-9.

106. Nicola, N.A. and D. Metcalf, *Binding of 125I-labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells.* J Cell Physiol, 1985. **124**(2): p. 313-21.
107. Menon, V., et al., *Flow cytometry protocols for surface and intracellular antigen analyses of neural cell types.* J Vis Exp, 2014(94).
108. Lazarus, A.H., et al., *Permeabilization and fixation conditions for intracellular flow cytometric detection of the T-cell receptor zeta chain and other intracellular proteins in lymphocyte subpopulations.* Cytometry, 1998. **32**(3): p. 206-13.
109. Allen, L.A., *Immunofluorescence and confocal microscopy of neutrophils.* Methods Mol Biol, 2007. **412**: p. 273-87.
110. Coppin, E., et al., *Flow cytometric analysis of intracellular phosphoproteins in human monocytes.* Cytometry B Clin Cytom, 2017. **92**(3): p. 207-210.
111. Christopher, M.J., et al., *Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice.* J Exp Med, 2011. **208**(2): p. 251-60.
112. Saito, M., et al., *Granulocyte colony-stimulating factor directly affects human monocytes and modulates cytokine secretion.* Exp Hematol, 2002. **30**(10): p. 1115-23.
113. Lie, A.K., et al., *Granulocyte colony-stimulating factor (G-CSF) dose-dependent efficacy in peripheral blood stem cell mobilization in patients who had failed initial mobilization with chemotherapy and G-CSF.* Bone Marrow Transplant, 1998. **22**(9): p. 853-7.
114. Cheng, A.C., et al., *Adjunctive granulocyte colony-stimulating factor for treatment of septic shock due to melioidosis.* Clin Infect Dis, 2004. **38**(1): p. 32-7.
115. Lee, A.W., et al., *Single-cell, phosphoepitope-specific analysis demonstrates cell type- and pathway-specific dysregulation of Jak/STAT and MAPK signaling associated with in vivo human immunodeficiency virus type 1 infection.* J Virol, 2008. **82**(7): p. 3702-12.
116. Moore, M.A., *The clinical use of colony stimulating factors.* Annu Rev Immunol, 1991. **9**: p. 159-91.
117. Rodriguez, R.M., et al., *Signal Integration and Transcriptional Regulation of the Inflammatory Response Mediated by the GM-/M-CSF Signaling Axis in Human Monocytes.* Cell Rep, 2019. **29**(4): p. 860-872 e5.
118. Lacey, D.C., et al., *Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models.* J Immunol, 2012. **188**(11): p. 5752-65.
119. Metcalf, D. and N.A. Nicola, *Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells.* J Cell Physiol, 1983. **116**(2): p. 198-206.
120. Castellani, S., et al., *G-CSF and GM-CSF Modify Neutrophil Functions at Concentrations found in Cystic Fibrosis.* Sci Rep, 2019. **9**(1): p. 12937.
121. Balazovich, K.J., H.I. Almeida, and L.A. Boxer, *Recombinant human G-CSF and GM-CSF prime human neutrophils for superoxide production through different signal transduction mechanisms.* J Lab Clin Med, 1991. **118**(6): p. 576-84.
122. Park, L.S., et al., *Interleukin-3, GM-CSF, and G-CSF receptor expression on cell lines and primary leukemia cells: receptor heterogeneity and relationship to growth factor responsiveness.* Blood, 1989. **74**(1): p. 56-65.
123. Richards, S., et al., *Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.* Genet Med, 2015. **17**(5): p. 405-24.

124. Yilmaz Karapinar, D., et al., *Congenital Neutropenia Patient With Hypomorphic Biallelic CSF3R Mutation Responding to GCSF.* J Pediatr Hematol Oncol, 2019. **41**(3): p. e190-e192.
125. Layton, J.E., et al., *Identification of ligand-binding site III on the immunoglobulin-like domain of the granulocyte colony-stimulating factor receptor.* J Biol Chem, 2001. **276**(39): p. 36779-87.
126. Bitard, J., et al., *Mutations in the immunoglobulin-like domain of gp190, the leukemia inhibitory factor (LIF) receptor, increase or decrease its affinity for LIF.* J Biol Chem, 2003. **278**(18): p. 16253-61.
127. Shakya, S., et al., *Optimized protocol for the preparation of single cells from cutaneous wounds for flow cytometric cell sorting and analysis of macrophages.* MethodsX, 2020. **7**: p. 101027.

7. Appendix

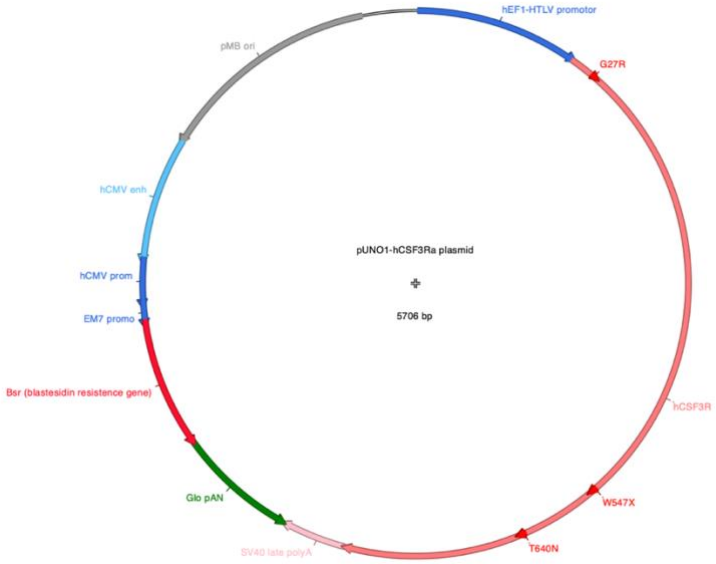

Figure 7.1 Illustration of the plasmid pUNO1-hCSF3Ra. The human *CSF3R* gene is shown in bright red with interesting mutations inserted as arrows. Figure was created using ApE (A plasmid editor).

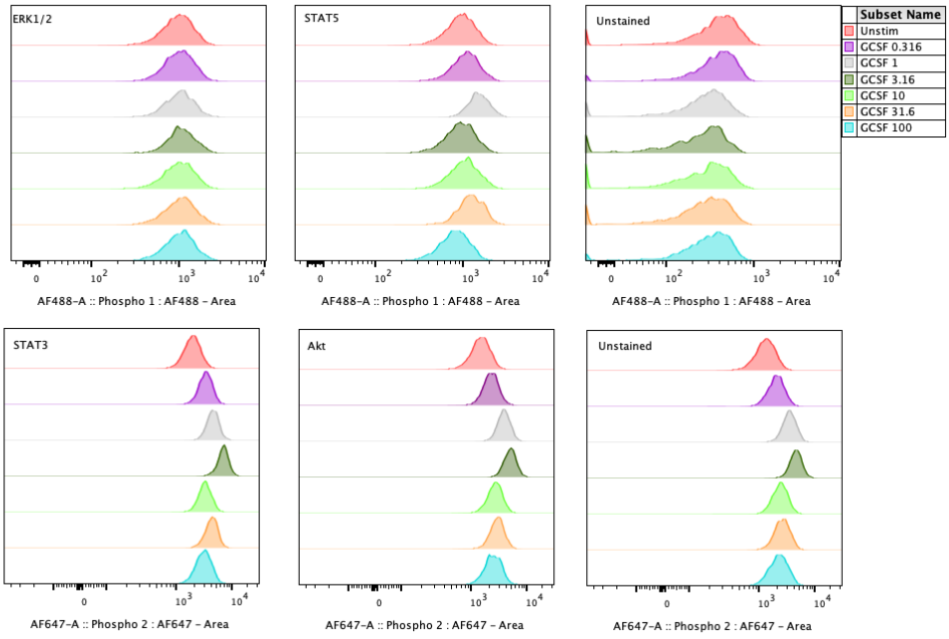

Figure 7.2 – Phospho-signal from PBL stimulated with six concentrations of G-CSF. Median fluorescent intensity (MFI) values for each of the four proteins are presented in a stacked formation with increasing G-CSF concentration from top to bottom. Unstained samples for both fluorochromes Alexa Flour 488 and 647 are also showed in the two plots on the right side of the figure. The G-CSF concentrations are color-coded with the legend shown to the right of the plots.

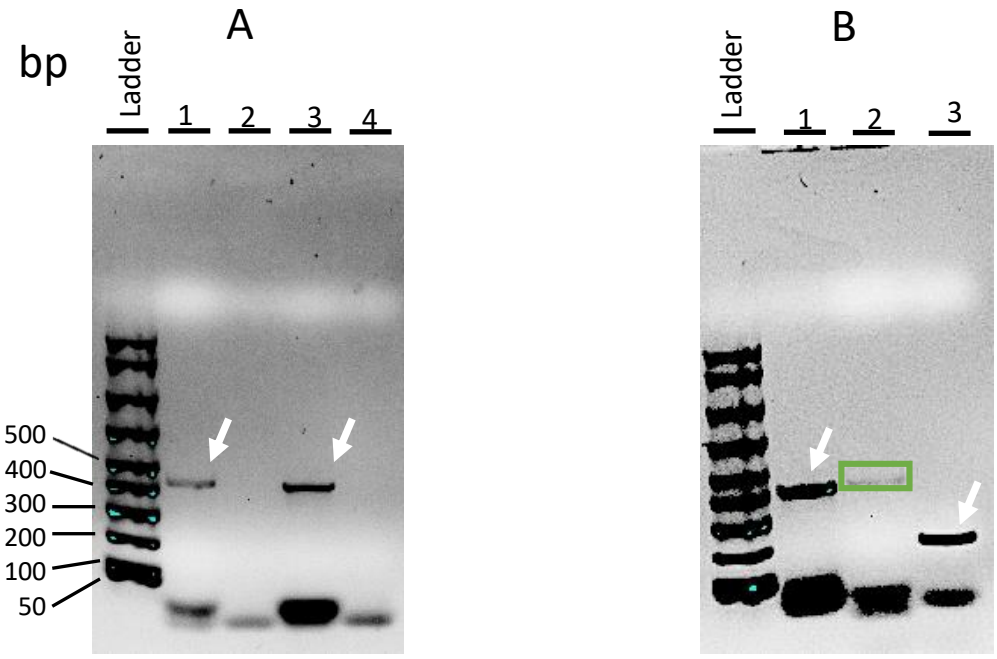

Figure 7.3 – Agarose gels with primers for CSF3R PCR amplification. The primers used for amplification of mutated *CSF3R* were tested to make sure they only produced one band. The PCR products were run on a 1% agarose gel. **A)** Two primer-pairs were tested, wells 1 and 3, and 2 and 4 contain PCR products from the same primers, with or without added betaine. A clear band are present for both parallel of the primer pair, represented with white arrows. **B)** Three primer pairs were tested on gel B. All three produced PCR products containing one band, but the PCR product in well 2 had a slightly less visible band, indicated with a green highlight.

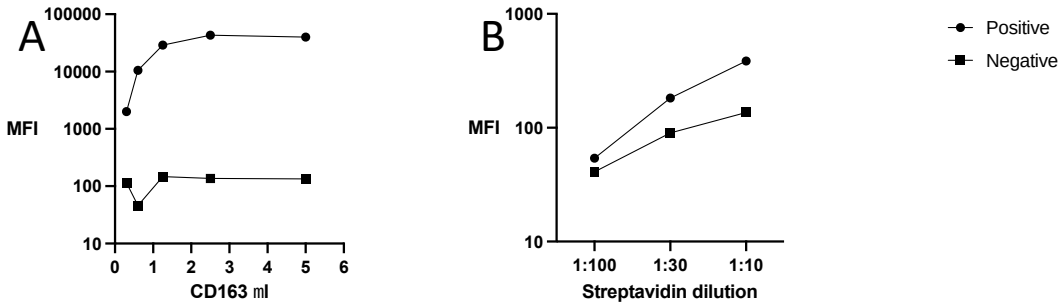

Figure 7.4 – Titration of CD163 and streptavidin PE. A) Tests on five anti-CD163 concentrations were conducted on PBMCs. The streptavidin amount was kept fixed for stained cells. The graph shows MFI values with logarithmic axis. **B)** Three streptavidin dilutions were tested on PBMCs. The anti-CD163 was kept fixed for the positive.