Single cell analysis of signal transduction in hematopoietic stem and progenitor cells from healthy donors

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This thesis is submitted in partial fulfilment of the requirements for the
degree of Master in Medical Biology – Medical Cell Biology

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March 2014

Acknowledgments

My first and sincere appreciation goes to Bjørn Tore Gjertsen, my supervisor
for support in all stages of this thesis. I would also like to thank him for being an open person
to ideas, and for encouraging and helping me to shape my own work and ideas.

I would like to express my deep gratitude and respect to Einar Kristoffersen, my co supervisor
whose advices and insight was invaluable to me. In addition, I would like to thank Guro
Melve at the Bloodbank for inspiring support and both Einar and Guro for providing
samples for the experiments.

I would like to thank all the people in the Hematology research lab for making me feel
welcome and for the nice working environment. A very special thanks goes to Andre, Jørn
and Sonia for your encouragement, support, advice and most of all your humor!
I would like to thank Sahba for helping me to carry out the animal experiments in the Animal
facility for your friendly and helpful attitude. My warm appreciation goes also to Kimberley
and Calum for assistance and advices.

Finally, I would like to pay special thanks to Sigbjørn, Sophie and my beloved daughter Jenny
for their support, love and encouragement during my years as a student besides my job.

Bergen, March 2014

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Abbreviations

ALDH	Aldehyde dehydrogenase
AML	Acute Myeloid Leukemia
ATM	ataxia-telangiectasia mutated
Bax	Bcl2 associated X protein
BM MNC	Bone marrow mononuclear cells
cAMP	cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
CDK	Cyclin dependent kinase
CFU	Colony forming unit
CHK2	Checkpoint kinase 2
CMP	Common myeloid progenitors
CST	Cytometer setup & tracking
Erk1/2	Phosphorylated Extracellular signal regulated kinase
FACS	Fluorescent activated cells sorting
FMO	Fluorescence minus one
GDP	Guanosine diphosphate
G CSF	Granulocyte macrophage colony stimulating factor
GM CSF	Granulocyte / macrophage colony stimulating factor
GMP	Granulocyte/macrophage progenitors
GTP	Guanosine triphosphate
GVDH	Graft versus host desease
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
IL 3	Interleukin 3
MAP	Mitogen activated protein
MDM2	Mouse double minute 2 homolog
MEK	MAPK ERK kinase
MEP	Megakaryocyte/erythrocyte progenitors
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MPP	Multipotent progenitors
PARP	Poly-ADP ribose polymerase
PI	Propidium iodide
Puma	p53 upregulated modulator of apoptosis
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma
RTK	Receptor Tyrosine Kinase
SCID	Severe combined immune deficiency
SOS	Son of Sevenless
SP	Side populations
Src	Sarcoma
Stat	Signal transducer and activator of transcription
Stat3	Phosphorylated Stat3 phospho-tyr705
Stat5	Phosphorylated Stat5 phospho-tyr694

Summary

Hematopoietic progenitor cells are essential source for supportive therapy in high dose chemotherapy and for allogeneic stem cell transplantation. Various sources of hematopoietic progenitor cells may vary in signal transduction responses. The interrogation of **single** cell signalling networks is a necessary step towards defining mechanisms that promote cancer progression, response to therapy and clinical outcome.

The tumor suppressor and transcription factor p53 has also been included in this work to study the level of p53 in hematopoietic progenitors. Mutations in p53 are common in a wide range of human cancers, but rare in hematopoietic malignancies. Mutations switch the cellular transcription program resulting in deregulation of the stress responses that normally maintain cell and tissue integrity.

This study describes the use of flow cytometry for expression of signal transduction in different material like bone marrow and leukapheresis products. Samples are collected from healthy donors recruited from medical students at Oslo University for bone marrow and donors selected by the Bloodbank, Haukeland University hospital for the allogeneic leukapheresis products. To be able to describe signal transduction in five different sub populations of CD34 positive cells, a panel of antibodies and a protocol for staining of surface and intracellular antibodies was established. The flow cytometry protocol was validated by an in vitro method; Colony Forming Unit assay and an in vivo xenograft transplantation using NSGS mice. The results demonstrate the heterogeneity in the CD34 positive populations also other groups has reported. Moreover we found a bimodal peak in the non-stimulated samples from the allogeneic leukapheresis products that we don't find in the healthy bone marrow samples that need further investigation. To sum up this work a panel of antibodies has been evaluated and a protocol has been established for signaling studies and evaluation of the different sub populations are still ongoing.

1. Introduction

1.1 Hematopoiesis and human hematopoietic stem cells

Hematopoiesis is the process by which hematopoietic stem cells self-renew, differentiates and mature to form the peripheral blood lineages. In the embryo and fetus it takes place in a variety of sites including the liver, spleen, thymus, lymph nodes, and bone marrow; from birth throughout the rest of life it is mainly in the bone marrow. Bone marrow is a very complex organ containing hematopoietic stem cells (HSC) and their descendants, as well as mesenchymal stromal and stem cells, osteolineage cells, sinusoidal endothelial cells and sympathetic neurons and the extracellular matrix[1]. Bone marrow is also a reservoir for several types of adult stem cells; hematopoietic-, mesenchymal- and neural stem cells. Normal hematopoiesis is a well-balanced process influenced by its microenvironment (stroma) and via cell-cell interactions. Regulation is mediated by intracellular signals of specific growth factors, cytokines and other environmental components. Hematopoiesis controls the number, self-renewal, proliferation, differentiation, quiescence and localization of the HSCs.

Normal peripheral blood includes mature myeloid cells (erythrocytes, monocytes, granulocytes, platelet-producing megacaryocytes and others) and lymphoid cells (B- and T-lymphocytes, NK- plasma- and dendritic cells and others). All blood cells are derived from multipotent hematopoietic stem cells (HSCs) through a succession of progenitors with decreasing limited potential of self-renewal under the control of growth factors and specific cytokines such as interleukins. HSC's can give rise to multiple but limited cell lineages unlike pluripotent cells that can give rise to many differentiated cells of an organism e.g. embryonic stem cells and totipotent stem cells that can give rise to all differentiated cells of an organism and extra embryonic tissues such as placenta e.g. fertilized eggs. HSCs normally resides as rare cells in the bone marrow in adult mammals together with a hierarchy of progenitors that become progressively restricted to several or single lineages. The HSC are usually characterized by two properties; 1) Self- renewal which is the ability to give rise to identical daughter HSCs without differentiation. 2) The capacity of multilineage differentiation[2]. Both these characteristics contribute to maintain the homeostasis of the blood system and allow the restoration of hematopoiesis after injuries, infections, blood diseases or surgery.

Figure 1: Hematopoiesis hierarchy
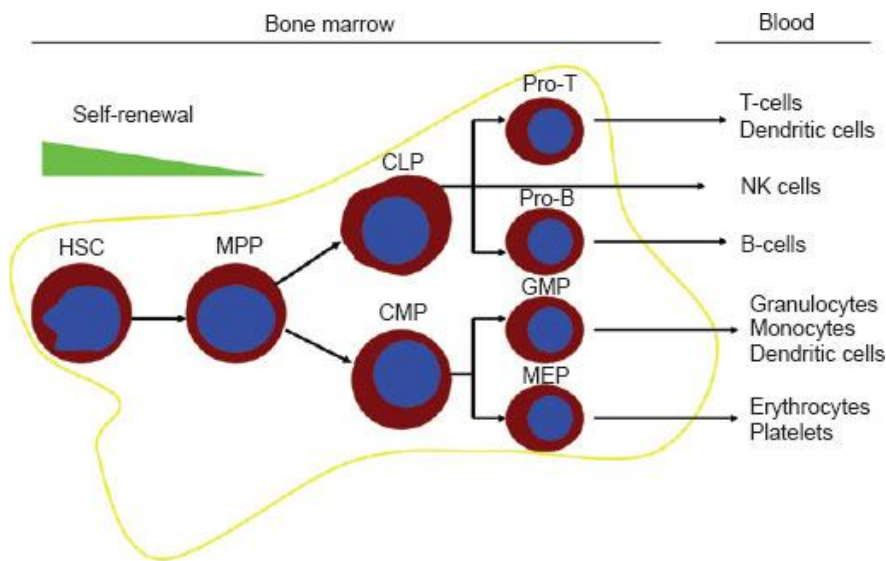
Figure 1: The origin of all blood cell in hematopoietic system is derived from **HSCs** that contain self-renewal capacity and give rise to MultiPotent Progenitors (**MPPs**) which lose self-renewal potential and start to differentiate into all multilineages. MPPs further give rise to common Lymphoid and Myeloid Progenitors, **CLPs** and **CMPs**. All these oligopotent progenitors differentiate into their restricted lineage commitment: CMPs progress to megakaryocyte/erythrocyte progenitors (**MEPs**) and granulocyte/macrophage progenitors (**GMPs**), CLPs give rise to T cell progenitors and B cell progenitors. Illustration adapted from [3]

The balance between differentiation and self-renewal of HSCs is organized by symmetric and asymmetric cell division [4, 5]. Under normal homeostatic conditions the number of HSCs is relatively constant. It is commonly assumed that this balance is achieved by asymmetric cell division by generation of two different daughter cells; one daughter cell which maintain the stem cell property and one becomes differentiated [5]. It is also assumed that asymmetric cell division plays a very important role in cancer disease particularly in connection with the "cancer stem cell hypothesis"[6] In some cancers, the transformation of a progenitor cell can lead to activation of self-renewal, a property of the "cancer stem cell" Several intrinsic (e.g. transcription factors) and micro-environmental(extrinsic) effects plays an important role in their maintenance and functional heterogeneity. The extrinsic growth factor/cytokine-mediated signal leads to a quick transcriptional response (in the order of minutes) and the central role of transcription factors in stem- cell biology suggests that alterations in the levels of these factors have an essential effect on cell fate. In most cases, the cytokines and growth factors that determine differentiation of hematopoietic stem cells into a particular lineage are well defined [7].

HSCs are widely used in therapy. Progress in the understanding of the cells of the hematopoietic system has provided a keystone in improving clinical hematopoietic cell transplants. Hematopoietic stem cell transplants are now routinely used to treat patients with cancers and other disorders of the blood and immune systems [8] .

1.1.1 Identification of HSCs and progenitors

Estimates have placed the number of HSCs at approximately 1-4 % of nucleated cells in adult bone marrow [9] and the limited number make the characterization and isolation challenging [10, 11]. The hematopoietic stem cell as a concept was first demonstrated by the two Toronto researchers Till and McCulloch [12]. In 1963 Till and McCulloch published their high impact paper on hematopoietic stem cells in Nature. Identifying and characterizing properties of HSCs began with studies in mice.

Human HSC's are functionally defined by their ability to retain the capacity to long term repopulate and multilineage differentiation after transplantation in severe combined immunodeficiency (SCID) mouse [13] or in the colony-forming assays where HSCs are plated at low cell density into semi-solid medium containing appropriate growth factors. These assay conditions allow for the proliferation and differentiation of hematopoietic progenitors into mature cell types, which can then be enumerated and characterized based on their specific morphology. Nonetheless, the hallmark of hematopoietic stem cells is their ability to reconstitute lifelong multilineage hematopoiesis in transplanted hosts, like in the NOD/SCID mouse [14]. Normal hematopoietic development is a complex biological process and in vivo models are required and the observation that immunodeficient mice can serve as hosts for human hematopoietic cells represented a major advance. Hematopoietic cells were shown to engraft in CB17-Prkdc SCID(severe combined immunodeficient)mice [15]. The SCID-hu mouse model for human hematopoietic stem cell engraftment was used to identify the Lin-CD34+CD90+ fraction of human bone marrow with repopulating activity [16, 17]. Improved engraftment levels were achieved using the NOD (non-obese diabetic) /SCID [18] and NOD/SCID/b2m$_{null}$ mouse strains [19]. Both strains have reduced NK cell activity compared with SCID mice and another benefit is polymorphisms in SIRPa that are responsible for mediating the enhanced engraftment potential of NOD/SCID mice over SCID mice by limiting macrophage phagocytosis of human hematopoietic cells through interactions with CD47 [20]

A great number of studies based on in vitro cell culture, xenotransplantation of hematopoietic stem cells and progenitors in immunodeficient mice and in pre-immune animal fetuses, have been carried out to identify the human HSC and uncover the hematopoietic hierarchy.[21]

To recognize HSC and progenitors from other cells can be a challenge. Morphologically they look very much like a lymphocyte and this makes them difficult to identify by morphology (size and shape). A heavily used method to identify HSC and progenitors in complex material like bone marrow, leukapheresis products, adipose tissue or cord blood is flow cytometry. The cells at each maturation stage carry a distinctive set of markers classified by the CD (cluster of differentiation) nomenclature and defined by flow cytometry. Researchers rely mainly on cell surface proteins, which serve as markers of HSC, progenitors and cells like leukocytes, megacaryocytes and erythrocytes. It has been proposed many different cell surface markers for HSCs during the years [17, 22-25]. The first cell surface monoclonal antibody used to identify human HSCs was CD34, a clone named anti-My-10 mouse monoclonal antibody, first called the My10 marker [26]. CD34 has been used in numerous clinical studies over the past decade to identify HSCs [17, 27, 28]. Although some studies using xenograft assays suggest that, similar to mice, human $CD34^-$ HSCs might exist [29, 30] but results among different studies using reported frequencies indicates that >99% of human HSCs must be $CD34^+$.

1.1.2 Antibodies for detection of HSC subpopulations in mouse and man

Numerous *CD34* mAb have become commercially available, clones like 8G12, 581, 561, QBEnd [31]. The CD34 marker is expressed on both immature hematopoietic stem cells able to self-renewal and progenitor cells with little or no capability to self-renew. CD34 was originally raised against KG-1a, a myeloblastic leukemic cell line [26]. CD34 is used as a marker for identification of stem- and progenitor cells in blood and bone marrow and as a marker in leukemia diagnosis and subclassification. CD34 is also an important marker in clinical transplantations.

In 1988, in an effort to develop a reliable set of markers to identify these cells, Irving Weissman and his collaborators at Stanford University, CA, focused on a set of protein markers on the surface of mouse blood cells that were associated with increased likelihood that the cell was a long-term HSC. Markers like Thy-1, Sca-1 and negatively selected for T-cells (CD4 and CD8), B-cells (B220), granulocytes (Gr-1) and monocytes(Mac-1) distinguish the mouse long-term (LT-HSC) and short-term (ST-HSC) hematopoietic stem cells (self-

renew-capable), and the Multipotent progenitors (MPP, low or no self-renew capability — the later the developmental stage of MPP, the lesser the self-renewal ability and the more of the mature markers) [32].

Four years later Irving Weissman and his collaborators focused on a set of protein markers on the surface of leukocytes[33]; Thy-1, known as CD90, CD34 and a set of Lineage positive markers to exclude mature leukocytes. Weissman group and collaborators proposed this set of markers to identify human HSCs and they also observed that when these cells begin to develop as distinct cell lineages the cell surface markers CD90 and CD34 are no longer identified and the positive lineage markers appear. The use of antibodies tagged with fluorochromes that selectively adhere to the receptors/antigens on the surface of the cell as a tool to identify stem cells is a very old technique named flow cytometry and has the ability to fluoresce or emit light energy when activated by an energy source such as a laser beam or ultraviolet light, first described by Wolfgang Göhde from the University of Münster, Germany in 1968. The combination of the chemical properties of fluorescence and unique receptor patterns on cell surfaces or intracellular properties to identify specific populations of stem cells makes this method widely used.

Table 1: Proposed HSC (self-renew capable) cell surface markers for mouse / human material like bone marrow, blood, cord blood and leukapheresis products. This markers are the most accepted ones in the past decade and has been used in several high impact publications [18, 23, 24] but there are also other markers and combinations [34, 35] available and it has always been a discussion if it is correct to use surface markers to identify undifferentiated HSCs [36]

Table 1: Antibodies for identification of hematopoietic stem cells and progenitors

Mouse	Human
CD34^{low}	CD34$^+$ [26]
CD38$^+$	CD38^{low} [37]
Sca-1$^+$	A human ortholog is not yet identified. [38]
C-kit (CD117)$^+$	C-kit (CD117)$^{-/low}$ [39]
CD90 (Thy-1)$^{+/low}$	CD90 (Thy-1)$^+$ [24]
Lin-	*Lin-

Examples of the markers commonly used to isolate human linage negative (lin−) cells are, CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD56, and CD66b and CD235a [40] *but this is dependent on the experiment performed.*

The lin- markers used in this thesis are CD2, CD3, CD14, CDCD16, CD19, CD56, CD235a.

1.1.3. Functional assays for detection of human hematopoietic stem cells

Other not so commonly used indicators for flow cytometry identification of HSCs are functional assays like the Aldehyde dehydrogenase (ALDH) and side populations. These are functional markers of HSCs unlike the surface markers that characterize the metabolic activity of the cell. ALDH are cytosolic enzymes involved in oxidation of intracellular aldehydes like alcohol, and retinol (Vitamin A) [41] and the activity are shown to be high in the HSC population. Side population are another functional marker used to identify HSCs.The protocol for identification of side population (SP) cells was originally established for murine bone marrow hematopoietic stem cells (HSCs), but it has also been adapted for human HSCs. It has been demonstrated that these two functional markers, ALDH and SP identify a heterogeneous group of HSC and must be examined further using additional surface markers. The techniques demands a lot of adaptions and adjustments for the actual cell type and experiment and there are some limitations with these techniques like it depends a lot on a healthy cell population as the functionality also reflects the health condition. In this thesis these techniques are not included.

Despite all the effort researchers have put in to develop reliable surface markers or functional markers to identify true repopulating human HSC there is still debate on which markers that are tied to the unique stem cell function [1, 36, 42].

1.1.4. Clinical use of hematopoietic stem cells

Hematopoietic stem cell transplants and therapy are routinely used to treat patients with cancers and other disorders of the blood and immune systems. Both autologous and allogeneic stem-cell transplantations have been important in the progress toward potential cure of leukemia, lymphoma, and other malignancies and it has also been a key approach toward the cure of some inherited hemoglobinopathy and bone marrow failure conditions. It was in the mid 1950´s they realized that bone marrow transplant could be used to treat diseases of the marrow and in 1968 the first successful transplant to treat an inherited immunodeficiency using a human leukocyte antigen (HLA) matched sibling. In 1975 E. D. Thomas et al reviewed the state of HSC bone marrow transplantation, and defined a set of critical issues in the field, including HLA matching, infectious complications, graft versus host disease (GVDH), etc. and showed clearly that one could use this therapy to cure people with leukemia. Later he reported curing half the leukemia patients who underwent transplantation when their disease was in chemotherapy-induced remission [43-45]. E. Donnall Thomas

shared the Nobel Prize in 1990 with Joseph E. Murray for the development of bone marrow transplantation in treatment of human diseases as leukemia.

Treatment of leukemia and lymphoma, characterized by abnormal proliferation of leukocytes, were among the first clinical use of HSCs. The patient's own cancerous hematopoietic cells are destroyed via radiation or chemotherapy, and then replaced with a bone marrow transplant, or with a transplant, leukapheresis product, of HSCs collected from the peripheral blood of a matched donor. The transplant is called an allogeneic transplant and a matched donor is typically a relative of the patient who has inherited similar human leukocyte antigens (HLAs) on the surface of their cells. It can also be an unrelated person with matched HLAs.

The heterogeneity of leukemia cells in individual patients has implications for the use and development of treatments that specifically affect the products of these gene mutations. This heterogeneity in the so-called leukemia stem cells could also complicate the targeting of these progenitors with specific antibodies or drugs.

1.1.4.1. Allogeneic – autologous transplantation

The two major transplant approaches in use are autologous (using the patient's own hematopoietic stem cells) and allogeneic (using related or unrelated donor hematopoietic stem cells). Autologous transplant allow the use of high dose chemotherapy with the intent of overcoming chemotherapy resistance in tumor cells. This therapy is followed by infusion of the patient's previously stored hematopoietic stem cells. It has also been used to attempt to reset the immune system in severe autoimmune disorders [46]. Allogeneic transplant approaches to cancer treatment also may involve high-dose therapy, but because of immunologic differences between the donor and recipient, complications like GVDH or graft failure can occur, other complications like immune effects and infections can occur in both autologous and allogeneic transplants. Another use of allogeneic HSCs transplants is in the treatment of hereditary blood disorders. Although autologous approaches are associated with less short-term mortality, many malignancies are resistant to mega-dose therapy alone and/or involve the bone marrow and then require allogeneic approaches for optimal outcome.

Recently, researchers have observed in animal studies and in vivo studies that hematopoietic stem cells appear to be able to form other kinds of cells, such as muscle, blood vessels, and bone [47]. If this can be applied to human cells, it may eventually be possible to use hematopoietic stem cells to replace a wider array of cells and tissues than once thought.

1.2. Signal transduction in hematopoiesis

Disorders of cell proliferation, differentiation, survival, and migration are fundamental events that can give rise to cancer, and abnormalities of signaling through enzyme-linked receptors have major roles in this type of disease.

A cell is highly responsive to specific extrinsic biochemicals such as growth factors, cytokines, chemokines and hormones in its environment and it has a pivotal effect on cell faith. Bone marrow stromal cells in close proximity to hematopoietic precursors produce many of the growth factors and cytokines needed in hematopoiesis. Intracellular signal-transduction cascades mediate the sensing and processing of a stimulus. The extracellular molecules that induce these rapid responses (in minutes and hours) are often not diffusible but are instead attached to receptors on the cell surface membrane. Aberrations in these pathways are a crucial cause for diseases such as cancer [6]. Cell signaling is dependent on cell surface receptors, either gated ion-channel receptors, G-protein coupled receptors or enzyme-linked receptors like the Receptor Tyrosine Kinase (RTK).

1.2.1 Important cytokines, growth factors and receptors in HSC differentiation and proliferation

Cytokines are central in determining lineage fate in the hematopoietic hierarchy by controlling proliferation and differentiation. They include interleukins (ILs), colony-stimulating factors (CSFs), interferons, erythropoietin (EPO) and thrombopoietin (TPO) [30] [31]. Hematopoietic cytokines are glycoproteins secreted by fibroblasts, monocytes/ macrophages, activated lymphocytes and endothelial cells [48].

A cytokine is an extracelluar molecule, usually a protein or a steroid hormone produced by other cells capable of stimulate growth, proliferation, differentiation and other cellular processes. In this process cell signaling is essential. The interaction of cell surface receptor extracellular domains with secreted ligands, e.g. cytokines, is essential to most types of cell signaling and cell-cell communication.

In the cell signaling process cytokines are also named ligands. Examples are cytokines that are able to bind to specific receptors on the surface of their target cells. The binding activates the receptor that in turn activates one or more intracellular signaling pathways. The downstream signaling cascade generates responses such as changes in enzyme activity and metabolism, gene expression, or ion-channel activity or altered shape and movement of the

cell. These cellular responses are able to control cell proliferation, differentiation, maturation, and survival.

It is a considerable overlap in function between many of the growth factors and cytokines and these soluble ligands can elicit a wide array of biological responses. Though many of them exhibit a functional overlay in their activity their biological actions may differ in subtle but important ways, for example cytokines can act on the same cell type to mediate similar effects. Both the redundancy and the definite differences can be explained by the sharing of common receptor subunits in combination with their own specific receptors [31]

Growth factors and cytokines are essential in regulating self-renewal, survival, proliferation, differentiation and function of hematopoietic cells and particularly G-CSF and IL-3 are key regulators of myeloid differentiation. [32] IL-3 is also referred to as multi-lineage colony stimulating factor and is produced by T cells and monocytes [30, 33]. IL-3 is not essential for the maintenance of normal hematopoietic steady-state functions [37] but are important for emergency hematopoiesis and immune response. IL-3 is usually produced in response to infections and injury, and serves to increase leukocyte numbers and enhance their state of activation [36]. G-CSF is produced by monocytes, fibroblasts and endothelial cells and is the major cytokine regulator of neutrophilic granulocytes [38]. G-CSF is used clinically to treat myelosuppression and its resulting immunodeficiency, as well as to mobilize hematopoietic stem cells and – progenitors from the bone marrow to peripheral blood, facilitating leukapheresis collection for transplantation [38].

1.2.2 Cell membrane bound receptors

Growth factors and cytokines usually require enzyme-linked receptors like the Receptor Tyrosine Kinases (RTK) or cytokine receptors and can mediate a direct and rapid effect. Multiple cytokines and growth factors utilizes common receptor subunits and common intracellular signaling pathways This shared use of signal transducers in the multichain cytokine receptor complexes can explain the functional redundancy of cytokines [56]. The membrane bound receptors contain an extracellular binding domain and an intracellular signal transduction domain. The intracellular domain has either intrinsic enzyme activity or will interact with a directly associated enzyme. The activation process of cytokine receptors is similar to members of the tyrosine kinase receptor family [33, 47, 48]. The family harbors tyrosine kinase domains that catalyze the transfer of phosphate groups to activate downstream signaling pathways. These receptors also allow protein-protein interactions via other structural

motifs like the SH2 and SH3 domains. The non-receptor tyrosine kinase members recruit intracellular tyrosine kinases to activate their signaling pathway

Kinase enzymes that specifically phosphorylate tyrosine amino acids are termed tyrosine kinases. The largest family of tyrosine kinases is the Src family [49]. They are all characterized by containing intracellular Src Homology 2 and -3 (SH2 and SH3) domains, held there partly by their interaction with transmembrane receptor proteins and partly by covalently attached lipid chains [49]. Another tyrosine kinase is Phosphoinositide 3-kinase (PI3 kinase) which phosphorylates lipids rather than proteins which serve as docking sites for signaling proteins [57].

Cytokine-type receptors are much like receptor tyrosine kinases, except they lack kinase activity themselves. They function by binding a non-receptor tyrosine kinase, thereby doing the same job with two separate proteins. The close association with non-receptor tyrosine kinases that transduce cytokine-mediated signals creates the functional equivalent of a receptor tyrosine kinase.

The cytokine receptor family is transmembrane receptors expressed on the cell surface. They are characterized by their lack of intrinsic tyrosine kinase activity and they are by the basis of common structural features grouped into six major families: Class I cytokine receptors, class II cytokine receptors, TNF receptors, IL-1 receptors, tyrosine kinase receptors, and chemokine receptors. The class I cytokine receptors, also known as the hematopoietin receptors, are the largest group among the cytokine receptor family. Most cytokine receptors share subunits, e.g. receptor for GM-CSF and IL-3 share subunit βc and Thrombopoietin (TPO) and G-CSF share a single chain subunit. The shared subunit is the signal transducing subunit and the structure is similar to other members of the cytokine receptor family. It both receives signals initiated by cytokine binding and is responsible for broadcasting signals to downstream signaling proteins [50]

1.3.3.Ras-MAP kinase pathway

One of the most common intracellular signaling pathways triggered by RTKs is the Ras-Mitogen Activated Protein (MAP) kinase cascade and it is a key signaling pathway involved in regulation of cell proliferation, differentiation and survival by the transmission of signals from membrane bound receptors. The Ras-MAP kinase signal transduction pathway consist of a kinase cascade that is regulated by phosphorylation and dephosphorylation by specific kinases, phosphatases, GTP/GDP exchange proteins, adaptor proteins and scaffold proteins.

The downstream cascade of enzymes terminates in the production of transcription factors [51]. Irregular signaling contributes to cancer and other human diseases and constitutive activation of MAP kinase proteins in experimental models has been shown to cause cell transformation and is implicated in tumorigenesis [52]. The Ras-MAP kinase pathway is often involved in sensitivity and resistance to leukemia therapy [52] and is estimated to be mutated in 30% of all cancers [53].

The Ras-MAP kinase pathway can be activated by growth factors which will phosphorylate the membrane-associated SH2 domain on the cell membrane receptor making it a docking site for the Guanine TriphosPhate (GTP)/Guanine DiphosPhate (GDP) exchange factor SOS (named after the Drosophila homolog protein Son Of Sevenless). This activates Ras, a monomeric GTPase localized and anchored at the plasma membrane. In normal quiescent cells Ras is GDP bound and inactive [54]. Ras function as a molecular switch and SOS converts Ras-GDP to Ras-GTP, and this in turn activates a range of downstream effector targets, of which Raf, a MAP kinase kinase kinase is the best characterized. Ras are activated by the majority of growth factor receptors and bind and recruit Raf to the cell membrane upon activation. Ras activates the first serine-threonine kinase in the MAP kinase cascade. Each of the three kinases in this cascade then activates the next by phosphorylating it. Because all three kinases in this pathway phosphorylate multiple substrates, the initial signal is amplified at each step. Each member of the Ras-Raf-MEK–Erk cascade has multiple isoforms with important biological consequences. If preferred isoforms are paired the most optimal amplification and transduction will find place [53].

Figure 2: The Ras-MAP kinase pathway
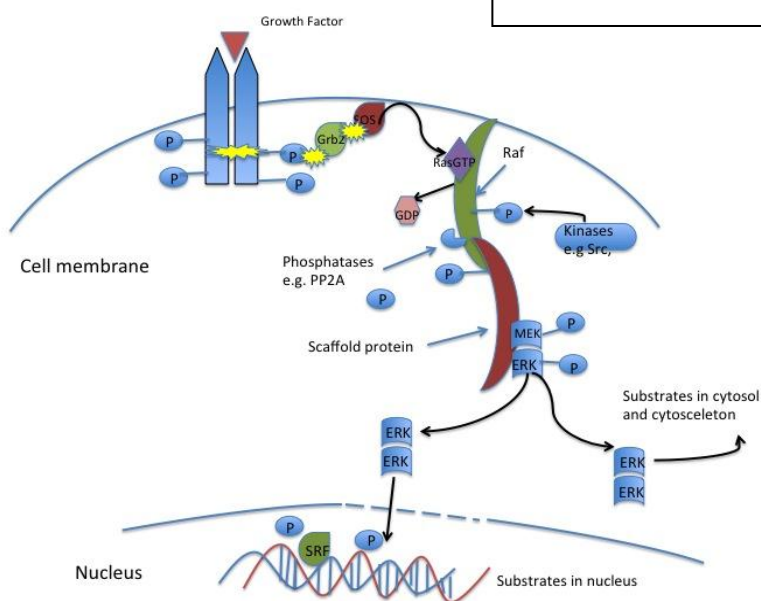
Activated ERK has many substrates in the cytosol [e.g.cytoskeletal proteins, phospholipase A2, and signalling proteins including tyrosine kinase receptors, SOS, signal transducer and activator of transcription proteins (STATs) and others.

ERK can also enter the nucleus to control gene expression by phosphorylating transcription factors such as Elk-1 and other Ets-family proteins. Grb2, growth-factor-receptor-binding protein 2; SOS and SRF-serum response factor

The three serine/threonine kinases are a MAP kinase kinase kinase (MAPKKK) like Rapidly Accelerated Fibrosarcoma (Raf), a MAP kinase kinase, like MEK and MAP kinase (MAPK) like Extra cellular signal Regulated Kinase (Erk1/2). There are four well-known MAPKs, the Erk 1/2, the c-Jun amino terminal kinases (JNK12/3), p38 kinases and Erk5. Generally, the Erk1/2 part of the pathway is activated by growth factors and cytokine ligands, whereas the JNK, p38 and Erk5 cascades are activated by inflammatory cytokines and different kinds of cell stress, like heat shock osmotic stress and ionizing radiation. The Raf kinase is required for phosphorylation of the MAP kinase kinase Mitogen associated /Extracellular regulated kinase-1 (MEK1). The MAP kinase Erk1/2 is the only known target for MEK1/2 phosphorylation [49]. Activated Erk1 and Erk2 has a variety of substrates and the number of Erk1/2 targets are high (> 60) [50]. Erk1/2 are also able to translocate to the nucleus and phosphorylate additional transcription factors, such as Elk-1, cAMP response element binding protein, Fos and globin transcription factor 1 and others [50]. Erk can also apply negative feedback by interfering with Ras activation by phosphorylation of SOS or Erk can phosphorylate Raf and MEK. This will alter the stability and activation of the signaling cascade [50]. Additionally certain protein phosphorylation events will serve to shut down or slow down the pathway which is a very intricate process involving fine tuning signaling originating from a growth factor or a mitogen [51].

1.4. JAK/STAT pathway

The JAK/STAT pathway is a main pathway for a wide array of cytokines and growth factors. The pathway is essential to hematopoiesis, immune development, mammary gland development and lactation, adipogenesis and other important cellular processes. The large family of cytokine receptors includes receptors for many kinds of local mediators and they are often associated with the family of cytoplasmic tyrosine kinases, Janus kinases (JAKs). JAKs appears to be a pivotal mediator of diverse cytokine signaling pathways [52, 53] [54].The JAK kinases are essential for phosphorylation of cytokine receptors, which results in establishment of docking sites on the receptors for such as STATs, Src-kinases and other signaling intermediates. The STATs are referred to as transcription factors or latent gene regulatory proteins because they only migrate into the nucleus and regulate gene transcription

after they have been activated. These transcription factors were originally described by Darnell and his colleagues [55].

Figure 3: Signaling via the JAK-STAT pathway.
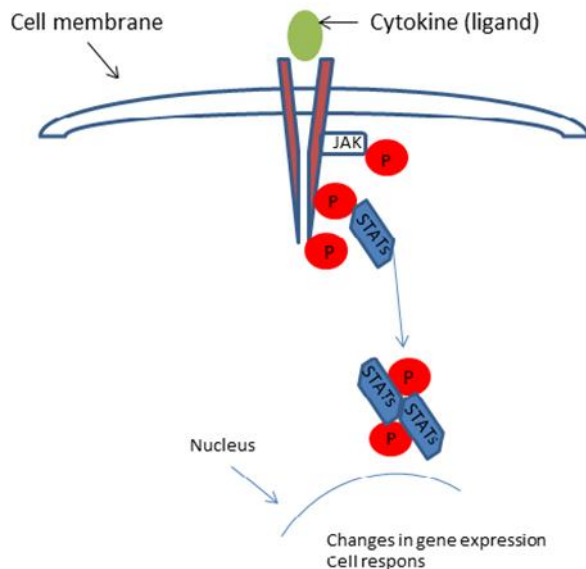
Figure 3. Ligand binding induces a conformational change of the cytokine receptor and allows phosphorylation of JAK proteins. Activated JAKs phosphorylate tyrosine residues in the receptor cytoplasmic domain and provide docking site for STAT proteins. Phosphorylated STATs dissociate from the receptor, dimerize and translocate to the nucleus where they modulate gene expression.

The recruitment of the JAK kinases appears to result in phosphorylation either by auto-phosphorylation or cross-phosphorylation by other JAK kinases or other tyrosine kinase family members. The increased JAK kinase activity will result in phosphorylation of tyrosine sites on the receptors and the receptors can then serve as docking sites that allow binding of other SH2-domain containing signaling molecules such as STATs, Src kinases, protein phosphatases and other adaptor signaling molecules. Some adaptor proteins can also bind to sites and couple cytokine receptors to the Ras-MAP-kinase signaling pathway. STATs contain a SH2 domain and a tyrosine residue that plays an important part in STAT activation [56]. Phosphorylation of the tyrosine residue is essential for the STATs dissociation and dimerization via their SH2 domain. The phosphorylation of STATs is known to occur immediately after the binding of a cytokine or growth factor to their receptors [56]. Activated STAT dimers translocate and accumulate in the nucleus where it activates transcription of target genes. The STAT family consists of 7 members, STAT 1-6. STAT5a and STAT5b are encoded by two separate genes. An important issue with STAT3 is its association with cancer. Constitutively activated STAT3 has been identified in many types of cancer, like head and neck, mammary, multiple myelomas and other hematopoietic [57].

1.5. p53 – The guardian of the genome.

p53 is one of the most important tumor suppressor proteins, containing transcriptional activation, DNA binding, and oligomerization domains encoded by TP53. The encoded protein is normally "off" but responds to diverse cellular stresses to regulate expression of a wide variety of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism and with this p53 play a critical role in maintenance of "cell integrity" under stress conditions. p53 is the most frequently inactivated tumor suppressor gene in human cancer and clinical studies have shown that p53 deletions or mutations are present in approximately 50% of human cancers. It was first believed to be an oncogene when first described in 1979 but convincing genetic and functional studies has revealed the characteristic properties of p53 [58]. A very descriptive example is the Li Fraumeni syndrome, where a genetic defect in p53 leads to a high frequency of cancer in affected persons.

Upon DNA damage, the sensor protein ataxia-telangiectasia mutated (ATM) is activated, which phosphorylates various downstream target proteins and induces the cell cycle checkpoint response. The general function of cell cycle checkpoint response is to detect damaged or abnormally structured DNA and to coordinate cell cycle progression with DNA repair. Their function is to slow or arrest cell cycle progression, thereby allowing time for appropriate repair mechanisms to correct genetic lesions before they are passed on to the next generation of daughter cells.

The best understood activity of p53, a common activation pathway and one of the first effects of p53 expression is after sensing DNA damage, activated ATM directly phosphorylates the tumor suppressor p53 at serine 15 within its amino-terminal transactivation domain. ATM also activates CHK2, a serine threonine kinase, which phosphorylates p53 at threonine 18 and serine 20 [59]. ATM also phosphorylates MDM2, an ubiquitin ligase that targets p53. These phosphorylations modify p53 and MDM2 and leads to transcriptional activation and stabilization of p53 [60]. p53 stimulates the expression of p21, an inhibitor of cyclin-dependent kinases (CDKs). CDKs are a family of protein kinases that are involved in the cell cycle as regulators and transcription regulation. CDKs and cyclin proteins are major control switches causing the cell to move from G1 phase to S or from M to G2. Activation of p21 will induce a delay or an arrest of the cell cycle to repair the DNA damage if it's repairable. If the DNA damage is too high or irreparable p53 will induce transcription of proapoptotic genes such as BAX, NOXA and PUMA to induce cell death [61, 62].

The importance of HSC´s for various transplantation therapies of incurable diseases such as leukemia's is obvious and continuous efforts to elucidate the precise functions of p53 as a main regulator of HSCs will remain crucial and provide an insight into new strategies for treating various disorders.

2. Aims

Hematopoietic progenitors have their malignant couterparts in myeloproliferative disease, myelodysplasias and various leukemias. Jak/STAT and MAPK/Erk pathways appear frequently involved in malignant hematopoieses [63] It is therefore of great interest to determine the signaling in normal hematopoiesis for comparison of rare or more more frequent malignant clones in bone marrow malignancies. The main aims of this thesis have been to develop a panel of antibodies for multiparametric characterization of signal transduction in the Jak/Stat and MAPK/Erk pathways and p53 in healthy hematopoietic stem cell- and myeloid progenitor populations. The multiparametric characterization of signal transduction was tested on different sources of hematopoietic progenitors, such as healthy bone marrow and leukapheresis products from donors for allogeneic stem cell transplantation.

The objectives have been:

1) Establish a protocol and a panel of antibodies with a set of surface markers and intracellular markers to define five different hematopoietic stem cell and myeloid progenitor populations and characterize cytokine response and signaling patterns in these populations in healthy individuals.

2) Establish a FACS sorting protocol for sorting of bone marrow derived mononuclear cell leukapheresis products .

3) For validation of stemness puroposes only: Examine the correlation between the different hematopoietic stem- and myeloid progenitor populations with ex vivo assay Colony Forming Unit (MethoCult) and in vivo xenotransplantation. To do/future perspectives: Signaling profile of ex vivo CFU and xenotransplantation.

3. Materials and Method

3.1 Cell lines and primary cells from healthy donors

The suspension wild-type TP53 MOLM-13 AML cell line , the promonocytic U937 suspension cell line and the suspension AML myeloblast cell line KG1a was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). MOLM-13 and U937 was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, Inc., St. Louis, MO, USA). KG1a was cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich). Cell culture media were supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (PAA Laboratories GmbH, Pashing, Germany), 50 UI/mL Penicillin – 50 μg/mL Streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich). The cells were kept in humidified incubators at 37°C with 5% CO_2 and observed under bright field microscope daily. Cell lines were maintained at 0.2-1.0×10^6 cells/mL for the duration of the experiment and split at a ratio of 1:5 three times a week.

Donors for allogeneic hematopoietic stem cell transplantation were voluntary recruited for donation of leukocytes (ethical approval, REK Vest, University of Bergen). Healthy individuals were recruited for donation of bone marrow by aspiration from puncture of posterior crista (ethical approval REK Sør-Øst, University of Oslo). Bone marrow from healthy donors were cryopreserved, stored and thawed according to procedures described below and in previous publications [ref].

3.1.1 Thawing of bone marrow mononuclear cells

Cells were thawed rapid in hands until almost defrosted. 1 mL of FBS was added dropwise and the vial was left at room temperature (RT) for 5 minutes. The cell suspension was then slowly transferred to a tube containing RPMI with 10% FBS and 80 units/mL[64] DNase (deoxyribonuclease I from bovine pancreas) (Sigma-Aldrich, St. Louis, cat. no. 2025), carefully mixed and incubated in RT for 10 minutes. The tube was centrifuged at 300G x 7 minutes, supernatant was removed and RPMI with 2 % FBS was added. Volume was adjusted according to the number of cytokines and timepoints in the stimulation assay. Cell suspension was carefully mixed and left in the cooling room (5-7°C) for 1 hour and 30 minutes before proceeding with the antibody staining procedure.

3.1.2. Cell line cryopreserving

Cells were counted and followed by centrifugation at 500g × 5 min. The supernatant was discarded and the pellet was dissolved in freezing medium (70% medium, 20% FBS and 10% DMSO at a concentration of 10.0×10^6 cells/mL. 1 mL of the well mixed cell suspension was transferred to a cryogenic vial (Sarstedt, Nümbrecht, Germany). The vials were stored at -80°C for 24 to 72 hours before transferring to a liquid nitrogen tank (-196°C) for long term storage.

Cells were counted by using a hemocytometer. All cell work was carried out under sterile conditions through the utilization of a laminar flow bench with a high efficiency particulate air (HEPA) filter.

3.1.3. Cell line thawing

Cells were rapidly thawed by heating the vial containing approximately 5.0×10^6 cryopreserved cells in hands until it was almost defrosted. 1 mL of medium was added dropwise, and the vial was left at room temperature (RT) for 5 minutes. The cell suspension was then transferred to a 25 cm² cell culture flask containing 9 mL of medium. After one day culturing the cell suspension was transferred to a 15 mL tube and centrifuged at 500g × 5 min. The supernatant was discarded to remove the cryoprotectant dimethyl sulfoxide (DMSO, Scahrlab S.L., Sentmenat, Spain), and the pellet was resuspended in 10 mL of fresh medium in the cell culture flask. The cells were cultured under normal growth conditions.

3.2. Flow cytometry

3.2.1 Antibody panel

Antibody panel for human bone marrow and leukapheresis product analyzed are presented. Flow cytometric analysis was performed in the UiB core facility for flow cytometry with instruments BD Fortessa and BD Aria Fortessa (both Becton Dickinson, La Jolla, CA, US) . The optimal concentrations for each antibody, empirically determined in pilot studies are shown in Table 1.

Table 2

Antibody	Clone	Fluorochrome	Cat no / Supplier	Ab pr 100 μl sample
Lineage depletion cocktail (Lin-)	*	FITC	22-7778/eBioscience (Santa Clara, CA, US)	15 μl
Live/dead marker (Anti-PARP, cleaved form)	F21-852	FITC	558576/BD Biosciences,(LaJolla, CA, US)	10 μl
CD34	8G12	PerCPCy5.5	347222/BD Biosciences	7 μl
CD38	HB7	Brilliant Violet 605	562665/BD Biosciences	5 μl
CD90	5E10	PECy7	561558/BD Biosciences	3 μl
CD123	9F5	Brilliant Violet 421	562517/BD Biosciences	5 μl
CD45RA	HI100	Brilliant Violet 510	304142 / BioLegend (San Diego, CA, US)	5 μl
p53	DO-7	PE	556534/BD Biosciences	15 μl
Erk1/2 (P-p44/42 MAPK)	pT202/Y204;E10	Alexa Fluor 647	4375 / Cell Signaling Techn.(Beverly, MA,US)	1,5μl
Stat3	pY705;4/P-STAT3	PE	612569/BD Biosciences	15μl
Stat5	pY694; 47	Alexa Fluor 647	612599/BD Biosciences	20μl

The **Lin-** antibody purchased from eBioscience contains markers for mature leukocytes and erythrocytes and contains CD2, CD3, CD14, CD16, CD19, CD56, CD235a

PARP (poly-ADP ribose polymerase) antibody is included as a live/dead cell marker for fixed cells. PARP is a DNA repair enzyme that is activated by DNA strand breaks and a target of the caspase protease activity associated with apoptosis. Proteolytic cleavage of PARP by caspase 3 into 24- and 89-kDa fragments inactivates the PARP enzyme and results in epitope unmasking for the anti-PARP clone F21-852. It has been proposed that inactivation of PARP directs DNA-damaged cells to undergo apoptosis rather than necrotic degradation, and the presence of the 89kDa PARP cleavage fraction is considered to be a marker of apoptosis.

CD34: Cells expressing CD34 are normally found in the umbilical cord and bone marrow, but is also expressed by small vessel endothelial cells. CD34 is a glycosylated transmembrane protein related to the proteins podocalyxin and endoglycan, and represents a well-known marker for primitive blood- and bone marrow-derived progenitor cells especially for hematopoietic and endothelial stem cells. The CD34 gene contain possible binding motifs for MYB, MYC, and ETS [65] transcription factors involved in cellular differentiation and fate determination [66, 67] . Even if CD34 is shown to be involved in homing of progenitor cells, its function mainly remain elusive.

CD38: Cyclic ADP ribose hydrolase (CD38) is a glycoprotein found on the surface of many immune cells. In humans, it is expressed on T cells, B cells, NK, myeloid, plasma, and dendritic cells. By functioning as both a cyclase and a hydrolase, CD38 mediates lymphocyte activation, adhesion, and the metabolism of cADPR and NAADP both of which are second messengers. The CD34+CD38- population of hematopoietic stem cells is thought to define the most multipotent cells (HSC)

CD90: Thy-1 is a glycophosphatidylinositol (GPI) anchored conserved cell surface protein. Thy-1 can be used as a marker for a variety of stem cells. Thy-1 is expressed on endothelial cells, smooth muscle cells, a subset of CD34+ bone marrow cells, and umbilical cord blood-, cardiac fibroblasts, and fetal liver-derived hematopoietic cells. It is considered a major marker of HSC together with CD34. In human HSCs, Thy-1 cells are all CD34 positive.

CD123: CD123 is also known as IL-3 receptor α-chain and is the primary low-affinity subunit of the IL-3 receptor. CD123 is expressed by low levels on monocytes, eosinophilic granulocytes, myeloid dendritic cells, and subsets of hematopoietic progenitor cells.

CD45RA: CD45RA is an exon 4 splice variant of the tyrosine phosphatase CD45. The isoform is expressed on resting/naive T cells, medullary thymocytes, B cells and monocytes.

p53: Antibody clone DO-7 reacts with the wild type and mutant type of the tumor suppressor p53 protein and recognizes an epitope between amino acids 1-45 of all known forms of the human p53 molecule.

Stat3: Signal transducer and activator of transcription (Stat) 3 is a 92-kDa protein that is phosphorylated in response to cytokine and growth factor stimulation, responding to phosphorylation with cytoplasmic-nuclear shuttling and thereby acting as a transcription factor. The anti-Stat3 clone used in this study detects only phosphorylations of Tyr705,

Stat5: The Stat5 phospho-Tyr694 antibody recognizes the regulatory tyrosine phosphorylation of human Stat5 protein

3.2.2. Protocol for stimulation with cytokines and staining for flow cytometry.

Stimuli used in the project were G-CS and IL-3 (final concentration 20 ηg/mL), both purchased from ProSpec Tany TechnoGene Ltd, Israel. The cytokines were frozen in aliquots in a -80°C freezer.

Density gradient centrifugation isolated mononuclear cells from healthy bone marrow donors (kind gift from Dr. June Myklebust, Radiumhospitalet) were thawed as previously described and rested one hour and 30 minutes in RPMI medium with 2% FBS in the cooling room (5-

7°C). Leukapheresis left-over cells received from the Bloodbank, HUS was resuspended in RPMI with 2% FBS and rested for one hour and 30 minutes in in the cooling room (5-7°C). Cytokines were added to cells in separate 15 mL tubes (Sarstedt, Germany) and for two time point measurements, 5 and 15 minutes. The suspension was mixed well and placed in a water bath at +37°C for 5 or 15 minutes. Signal transduction was halted by fixing the cells with a final concentration of 1.6% paraformaldehyde (PFA) diluted from 16% PFA (Alfa Aesar GmbH, Germany) for 15 minutes at room temperature. Cells were washed once and supernatant was removed. Cells were resuspended in PBS and Octagam (Octapharma AG, Switzerland) to a final concentration of 50 ug/mL was added to prevent nonspecific binding of antibodies and were incubated for 10 minutes in room temperature. Cells were centrifuged and washed once and resuspended in staining buffer (1xPBS with 0.5% BSA) and then stained with pre-determined saturating concentrations of fluorochrome-conjugated surface markers, 30 minutes in the dark in room temperature. After two washing steps the supernatant was removed and the cells were permeabilized with ice-cold 100 % MetOH (Sigma Aldrich) for 30 minutes at +4 °C. Cells were washed twice to remove remaining MetOH and resuspended in staining buffer and stained with pre-determined saturating concentrations intracellular signaling antibodies for 30 minutes. After 2x wash in staining buffer the cells were resuspended in staining buffer.

Figure 4. Experimental outline for detection of intracellular signaling events in single cell hematopoietic stem and progenitor populations.

Stimulate sample (BM, LP, CB single cells)

Fix cells with PFA, wash and stain for surface markers

Permeabilize with MetOH and stain for intracellular markers
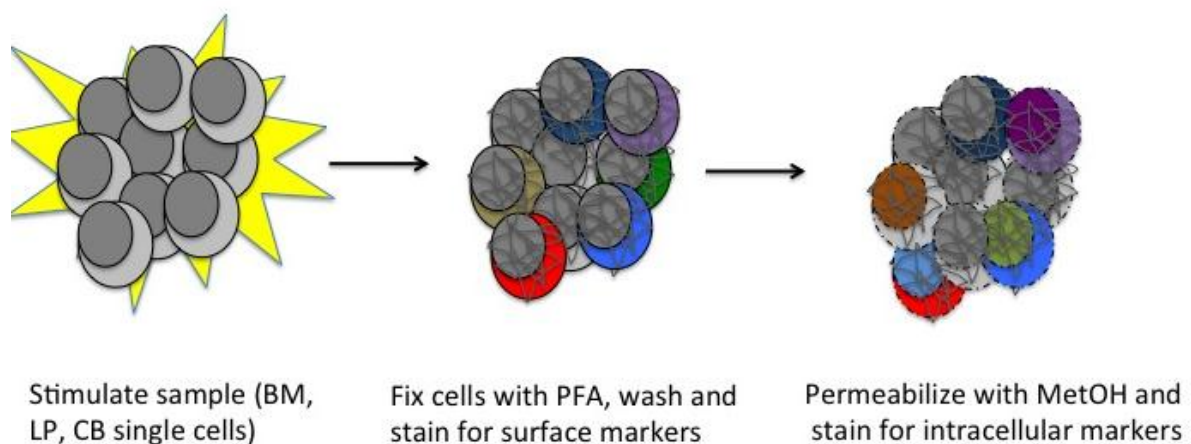
Antibody expression levels were quantified using the BD LSR equipped with four lasers (488 nm-50mW, 561nm-50mW, 640nm-70mW and 405nm-40mW) and 15 fluorescence detectors. Cytometer Setup & Tracking (CST) quality control was performed on the flow cytometer each day during analysis period. Fluorescence data was compensated at the time of acquisition using a compensation matrix set up using BD FACSDiva acquisition software (BD Biosciences). The compensation controls are BD Comp beads Anti-mouse Ig, κ (Cat no 552843, BD Biosciences) single stained with all the antibodies in the panel and a tube with unstained cells. FCS 3.0 data files were then uploaded to FlowJo (Tree Star,Ashland,OR,US) for further analysis and gating out of debris, non- viable cells and doublets. Non-viable cells and doublets were excluded based on PARP staining (viability staining) and forward scatter height versus area plots, respectively. Surface- and intracellular marker expression levels were calculated by subtracting the median fluorescence intensity (MFI) of non-stimulated cells from the MFI of cells stimulated with cytokines.

3.2.2.1. Compensation controls:

To account for spectral overlap between fluorochromes, anti-mouse Ig, κ particles, which bind any mouse κ light chain-bearing immunoglobulin, and negative control BD CompBeads (BD Biosciences), which have zero binding capacity, were used to set compensation levels. To prepare CompBeads for collection, one drop of anti-mouse Ig, κ and one drop of negative BD CompBeads, 100 µl of staining buffer and identical µl of fluorochrome-conjugated antibody as for the sample were added to a 5 mL FACS tube and incubated at RT for 30 minutes. After incubation, beads were then washed in 3 ml FACS buffer and centrifuged at 400 G for 10 minutes. The supernatant was aspirated and beads were resuspended in 300 µl FACS buffer for acquisition. 5000 events were acquired on the LSR Fortessa flow cytometer, while gating on positive and negative populations for compensation matrix and automatic compensation matrix setup in the FACS Diva software version 6.2.

Figure 5: Outline of the basic features of the cuvette flow cell.
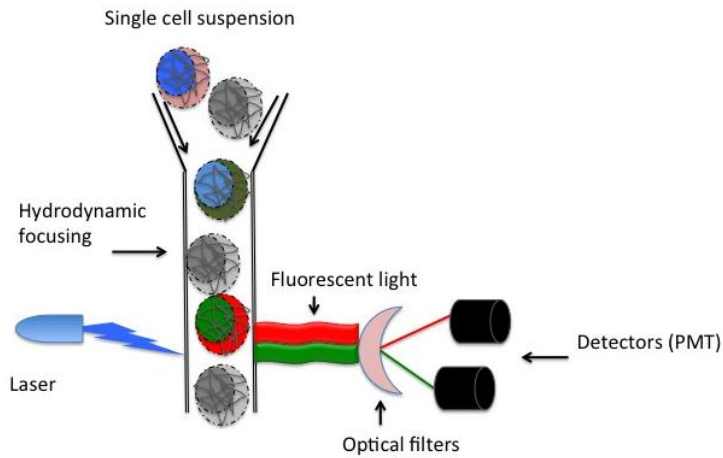
Figure 5: The sample in a single cell suspension will pass by the laser interrogation point one by one due to the hydrodynamic focusing and pressure. The central core were the sample is injected are enclosed by an outer sheath that contains faster flowing fluid caused by different pressure.

The fluorescent signal from the cells will pass by filters of a set range of nanometers and the filtered fluorescent signal will be detected by Photo Multiplier Tubes (PMT). The signal will be amplified, converted and transmitted to the computer. The data are stored in a Flow Cytometry Standard (FCS) format.

3.3. Fluorescent Activated Cells Sorting (FACS) for xenotransplantation and Colony forming unit assay.

Leukapheresis left-over product received from the Bloodbank, HUS was resuspended in staining buffer (1xPBS with 0.5% BSA) in room temperature under sterile conditions and cell number was determined using a hemocytometer. After treating the cells with Octagam (Octapharma AG, Switzerland) to a final concentration of 50 μg/mL for preventing non-specific binding of the antibodies, the single cell suspension was stained in 15 mL tubes with identical pre-determined saturating concentrations surface markers as for the intracellular signaling assay.

Antibodies in this panel are identical to the ones used for the signaling assay with phospho-antibodies.

For 500 μL sample the following amounts were used:

Lin- FITC   50 μL

CD34 PerCPCy5.5   35 μL

CD38 Brilliant Violet 605   30 μL

CD90 PECy7   15 μL

CD123 Brilliant Violet 421   25 μL

CD45RA Brilliant Violet 510   25 μL

The cells were incubated with the antibodies in room temperature for 30 minutes in the dark. After 2 x wash and centrifugation at 350 G in 10 minutes to get rid of unbound antibodies the cells were resuspended in staining buffer. Cell sorting on a BD FACS Aria SORP II configured with four lasers (488nm-100 mW, 532 nm-150mW, 640 nm-40 mW and 405 nm-50 mW) and 15 fluorescence detectors was performed on the four following populations:

Hematopoietic Stem Cells: **HSC** - CD34+CD38-CD90+CD123-CD45RA- (Lin-)
Multipotent Progenitor: **MPP** - CD34+CD38-CD90-CD123-CD45RA (Lin-)
Common Myeloid Progenitor: **CMP** - CD34+CD38+CD90-CD123+CD45RA- (Lin-)
Granulocyte-Macrophage Progenitor: **GMP** - CD34+CD38+CD90-CD123+CD45RA+ (Lin-)

The fifth population; Megakaryocyte-Erythroid Progenitor: **MEP** - CD34+CD38+CD90-CD123$^{lo/neg}$CD45RA- (Lin-) was not included of practical reasons, the BD FACS Aria are able to sort only four populations simultaneously in 5 mL FACS tubes and the sample amount was limited.

Compensation controls were performed and includes BD Comp beads Anti-mouse Ig, κ (Cat no 552843) single stained with all the surface markers in the panel and a single tube with unstained cells, similar to the set up for analysis of intracellular signaling in the five stem and progenitor populations. A live/dead marker (Propidium Iodide) in a single tube was included in the sort set up in addition to excluding necrotic and apoptotic cells and debris by gating on the Forward scatter (Area) versus Side scatter (Area) dot plot. Unstained cells were included as negative control. Doublets and other events with unwanted light scatter properties were gated out in a forward scatter width versus area plot. Cytometer Setup & Tracking (CST) quality control was performed daily before setting up for cell sorting. Prior to sorting the cell

suspension was filtered through a 40 μm cell strainer (BD Falcon, Becton Dickinson Labware Europe, Le Pont de Claix, France). The sorted cells were collected and the cell number was marked on the different sterile 5 mL tubes (Becton Dickinson Labware Europe, Le Pont de Claix, France) for further experiments. All cell work was carried out under sterile conditions except for the sorting. The sorter is not placed in a sterile environment.

The Aria was set up with a 70 μm nozzle and 90 Pounds per square inch (psi) pressure that makes it possible to run about 10 000-30 000 events per second for rapid enrichment with acceptable purity of the sorted populations (result: >95% purity). The air pressure forces the sample cells through an optically gel-coupled cuvette flow cell, special designed for the BD Aria model that ensures lasers to be precisely focused on the single cells in the sample stream. The flow cell optimizes the resolution and sensitivity of the fluorochromes and improves the collection efficiency by the no-need for alignment of the lasers on a daily basis or when the sorting is interrupted by a clog in the sample unlike the jet-in-air FACS cell sorters.

3.4 In vivo evaluation of human hematopoiesis through xenotransplantation of purified hematopoietic stem cells from G-CSF stimulated healthy donor leukapheresis material.

Stem cell activity was evaluated by the in vivo assay—transplantation of human cells to immunodeficient NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3 (NSGS) mice. These mutant mice combine the NOD/ShiLtJ background, the severe combined immune deficiency mutation (SCID) and IL-2 receptor gamma chain deficiency. As a result, the NSGS mice lack mature T cells, B cells, or functional NK cells, and are deficient in cytokine signaling and this should lead to better engraftment of human hematopoietic stem cells and peripheral-blood mononuclear cells of the myeloid lineage.

The four populations sorted by the FACS cell sorter (HSC, MPP, CMP and GMP) were left over night in staining buffer before transplantation. Next morning the different tubes were checked for viability on the cell sorter. Cells were spun down and washed once with sterile 9 mg/mL NaCl (Fresenius Kabi, Germany). The supernatant was removed and 100 μL NaCl 9 mg/mL added to the cells. The cell suspensions were transferred to insulin syringes (U-100 Insulin 0,5 mL, B.Braun, Germany) on ice and transported to the animal keeping facility, University of Bergen.

The cells from the four different populations were injected through the tail vein and a negative (non- injected) mouse was included in the experiment. The mice were treated according to the animal facility guidelines, and experiments approved by the national animal ethics committee. Stem cell activity was not evaluated from healthy donor bone marrow due to limited material.

Peripheral blood samples were taken from the facial vein with a lancet and a small blood collecting tube containing EDTA as anticoagulant every second week from week 6 after injection. At week 6 peripheral blood was incubated with anti-human CD45 30 minutes in the dark, room temperature. After incubation erythrocytes were lysed with PharmLyse (cat no 558049, BD Biosciences) in 8 minutes. Cells were then washed twice with PBS and resuspended in 250 μL PBS. The samples, included the negative control were analyzed using the same BD LSR Fortessa as for the signaling experiments. Blood samples were taken every second week from week 6 and stained for CD45.

3.5 In vitro evaluation of the four populations, HSC, MPP, CMP and GMP: Hematopoietic Colony Forming-Unit (CFU) assay.

Prospectively FACS sorted cell populations from leukapheresis (HSC, MPP, CMP and GMP) were plated in duplicate in 0.4 mL methylcellulose-based H4433 Methocult® medium (StemCell Technologies Inc., Vancouver, Canada) in wells on a 24-well plate. Remaining wells were filled with 0.4 mL sterile 9 mg/mL NaCl to prevent the Methocult medium from drying out and secure optimal humidity during incubation period. Plates were incubated at 37°C in a humidified incubator at 5% carbon dioxide. After 8 days, the number of colonies was examined. After 21 days the number of colonies was counted and images were taken by using Zeiss Axio Vert.A1 an inverted microscope with camera. The CFU assay was repeated once and this time the colony-forming units (CFUs; CFU-granulocyte and macrophage, burst-forming units–erythroid, and colony-forming units-mixed) were counted after 10 days using the inverted microscope. The four populations were plated in duplicate as the first time.

3.6. Statistical analysis

Statistical analysis was performed by using the GraphPad PRISM® (version 6.0, GraphPad Software, Inc., La Jolla, CA, USA) software. Data were expressed as mean ± standard deviation (SD)

4. Results.

4.1 Establishment of an antibody panel for evaluation of signal transduction in hematopoietic stem cells and myeloid progenitors.

To be able to study human normal bone marrow signal transduction profiles in defined sub-populations five well characterized panels of direct conjugated antibodies for human hematopoietic stem cells and myeloid progenitors were established [23]. The five sub populations were hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMP), granulocyte- macrophage progenitors (GMPs), and megakaryocyte- erythroid progenitors (MEPs). The panel of antibodies was adapted for analysis on a BD Fortessa flow cytometer.

4.1.1 Building an antibody panel.

The specific clones of the antibodies used for this particular protocol with surface markers and intracellular signalling antibodies have been described by others [23, 68]. To combine the ideal fluorochromes a spectral viewer tool and Brightness Index from BD Biosciences were used to determine the best fluorochrome combination. To minimize spectral overlap fluorochromes were spread over multiple lasers. The specific fluorochromes were chosen according to antigen ranking.

1: Necessary antigens: Basic phenotypes like Lin-, CD34, CD38, use fluorochromes with spill over problems

2: Important subset antigens like CD45RA, CD123, CD90 with fluorochromes that perform well with little bleedthrough problems.

3: Random antigens, intracellular antigens or dim antigen density like p53, Stat3, Stat5 require the brightest fluorochromes with the least spill over problems like PE and AF647

These criteria are important for choosing the optimal fluorochromes for the antibodies in the panel but the most significant limitation may be commercial availability of fluorochromes conjugated to defined clones.

4.1.2 Titration of antibodies

Titration of the antibodies was performed to obtain optimal balance of brightness between the fluorochromes. Correct titrated antibodies will also influence on the degree of bleedthrough

between emission of the various fluorochromes and not just the intended signal. The sample material used for titration was both primary cells like peripheral blood, leukapheresis product and cell lines depending on the actual antibodies. For CD34 and CD90 positivity the cell line KG1a was used in addition to leukapheresis product. For p53 signal MOLM-13 was used. For phospho-Stat 3/5 and Erk1/2 the cell line U937 was used. For all other surface antibodies peripheral blood and leukapheresis product were used. The last evaluation was done when the antibodies was combined in the panel and tested out on live cells and compared to fixed and permeabilized cells.

4.1.3 Fixation and permeabilization for intracellular antibodies

Signal intensity is affected not only by titration of antibodies but also by a good fixation and permeabilization optimization to ensure accessibility of intracellular antigens and preservation of surface markers. The timepoint and concentrations, choice of cytokines and fixation and permeabilization agents have already been performed in-house, and it is confirmed by studies of Gary Nolan lab at Stanford University, CA, US [69, 70].

The fixation and permeabilization time was tested on fresh leukapheresis product, 5×10^6 /mL. 5-, 10-, 15- and 30 minutes fixation were tested and surface markers were stained for right after fixation.10 min fixation and 30 min permeabilization in the fridge at approx. +4°C were optimal for the expression of the surface - and intracellular antibodies of this panel.

4.1.4 Gating controls

In addition to compensation controls, an unstained control and a Fluorescence Minus One (FMO) control for CD38 was included because there was no clear division between positive and negative populations [71]. An immunoglobulin isotype control for total p53 protein was tested but no difference in level of staining from the unstained sample was observed. The isotype control was not included in the protocol for staining. The FMO control identify contribution of spillover into one particular detector and facilitate precise gating on negative/positive population boundaries, isotype controls can help identify staining issues and unstained controls show the background or autofluorescence of the cells used.

4.1.5. Controls for the signal transduction profile

Since one of the purposes of this study was to evaluate normal samples and response to treatment with cytokines, untreated or unstimulated cells provided the best control for evaluating background staining. Unstimulated control cells were included in the protocol and were handled identical to the cytokine stimulated cells. This should take into account the

unique background characteristics of each antibody as well as the basal phosphoprotein expression level. To control the functionality of the cytokines used in the study PBMC and three cell lines were used, U937, Molm-13 and KG1a and expected result was achieved.

## 4.2 Phosphoprotein response to cytokine treatment of subpopulations of CD34 positive human hematopoietic cells.

4.2.1 Comparing signaling patterns of non-stimulated sample from healthy bone marrow and G-CSF treated donor leukapheresis product

A staining protocol for flow cytometry was developed to allow for detection of surface marker expression and simultaneous intracellular phosphoprotein detection in five defined sub populations. Flow cytometry was the method of choice because it is ideal for detection of signaling response mediated by protein phosphorylation in subsets of complex cell mixtures such as bone marrow, cord blood and leukapheresis products. The subsets were: hematopoietic stem cells **HCS´s**, multipotent progenitors **MPP´s**, common myeloid progenitors **CMP´s**, granulocyte- macrophage progenitors **GMP´s** and megakaryocyte-erythroid progenitors **MEP´s**.

To evaluate the direct effects of the cytokines, G-CSF and IL-3 on the five sub populations, cells were treated with G-CSF or IL-3 separately in 5 or 15 minutes. Cells were then fixed with paraformaldehyde to preserve the activation state.

Flowcytometric analysis of bone marrow and leukapheresis samples revealed a heterogeneous response within the different sub populations both to the two different cytokines and the two different timepoints as shown in figure 3. These findings are confirmed by Gibbs and co-workers [23]

Figure 6. Gating strategy for bone marrow mononuclear cells.
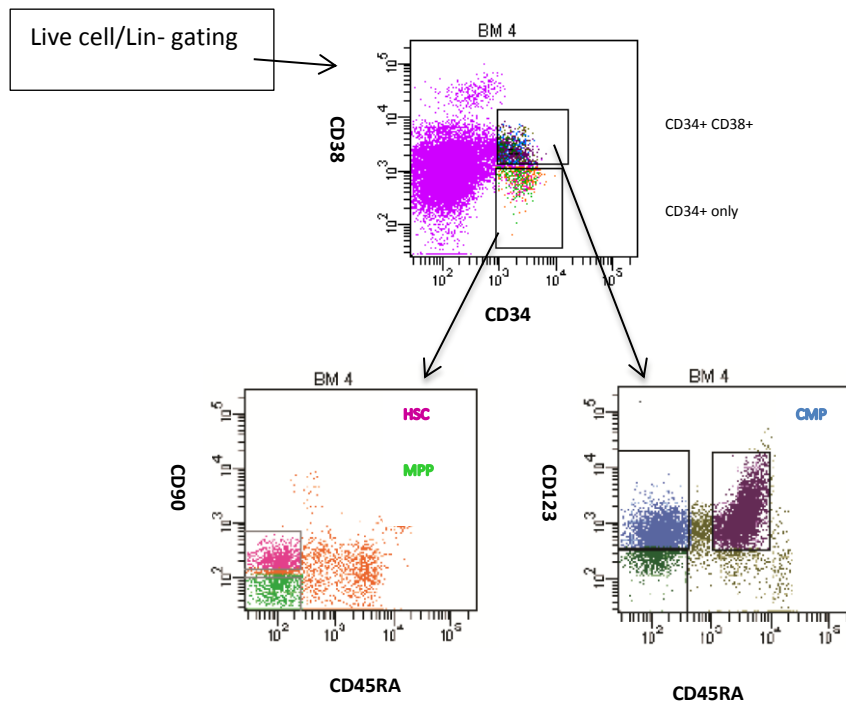
Figure 6. BM MNC was analyzed for the expression of lineage markers CD34, CD38, CD90, CD45RA and HLA-DR by flow cytometry. The upper dot plot is gated on lineage-negative (Lin-) viable events whereas below left dot plot is gated on the CD34 positive CD38 negative events and show the distribution of the HSC and MPP populations. The below right dot plot is gated on CD34 positive CD38 positive events and show the distribution of the CMP,GMP and MEP populations.

Figure 7. Flowcytometric analysis of intracellular signal transduction reveals heterogeneity in subpopulations of CD34+ hematopoietic cells that respond directly to G-CSF and IL-3

Normal bone marrow - MEP
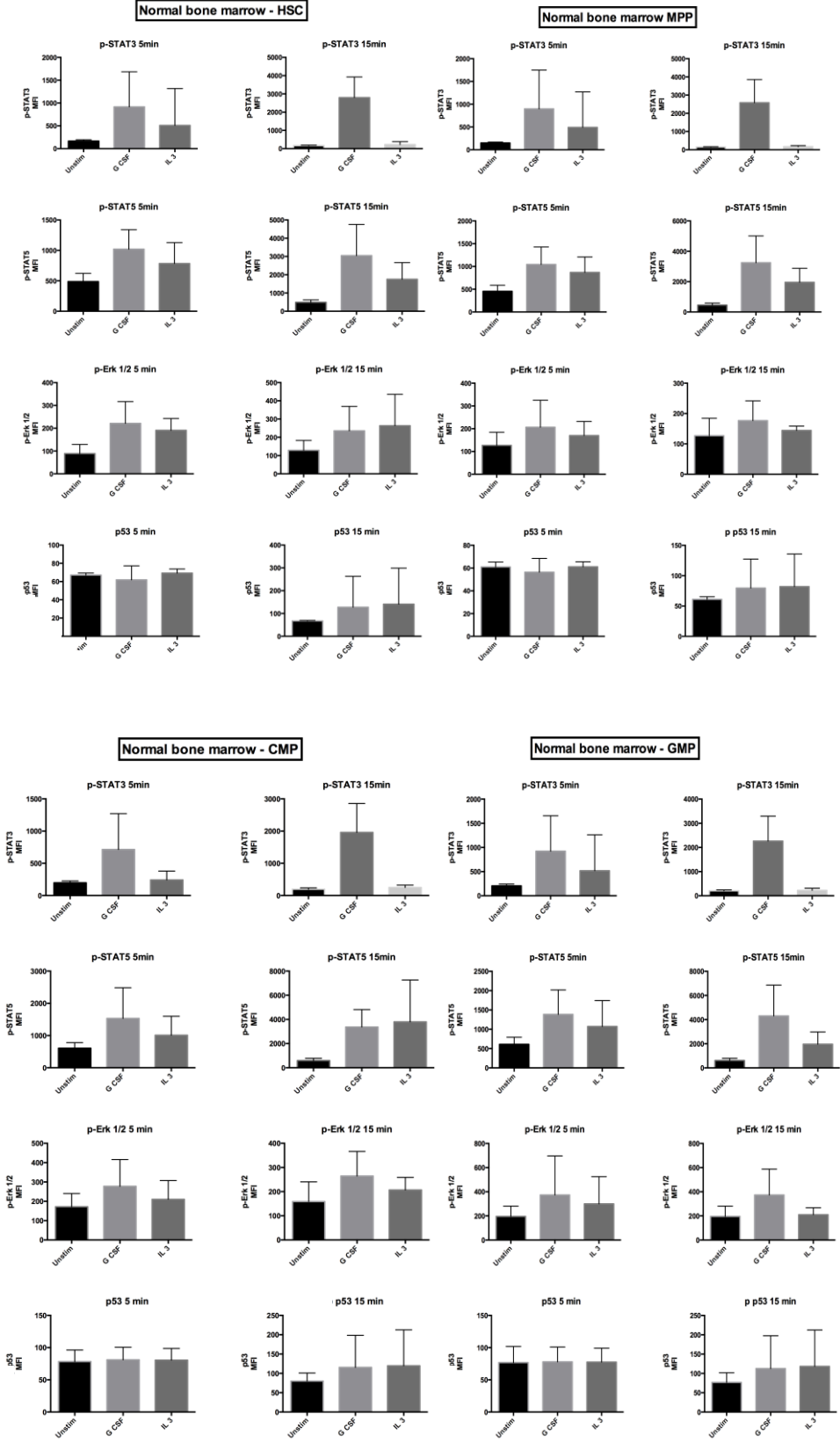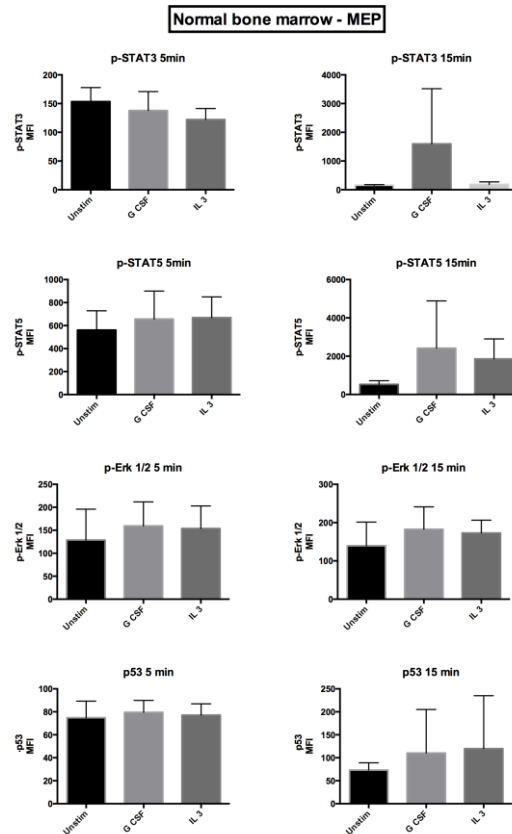
Figure 7:

Hematopoietic Stem Cells (**HSC)** - CD34+CD38-CD90+CD123-CD45RA- (Lin-)

Multipotent Progenitor (**MPP)** CD34+CD38-CD90-CD123-CD45RA (Lin-)

Common Myeloid Progenitor (**CMP)** CD34+CD38+CD90-CD123+CD45RA- (Lin-)

Granulocyte-Macrophage Progenitor (**GMP)** - CD34+CD38+CD90-CD123+ CD45RA+ (Lin-)

Megakaryocyte-Erythroid Progenitor (**MEP)** - CD34+CD38+CD90-CD123lo/neg CD45RA- (Lin-)

Mean fluorescence intensity was calculated and compared to untreated controls. Results are displayed as bar graphs of the mean ± SD of five separate bone marrow donors. Graphs show results both for 5 and 15 minutes stimuli to all five populations

Figure 8. Bone marrow mononuclear cells, represented by donor 4. Heatmap of the transformed medians by first row of cells responding in each stem and progenitor sub population. The heatmaps present the variations in the data files via variations in color.
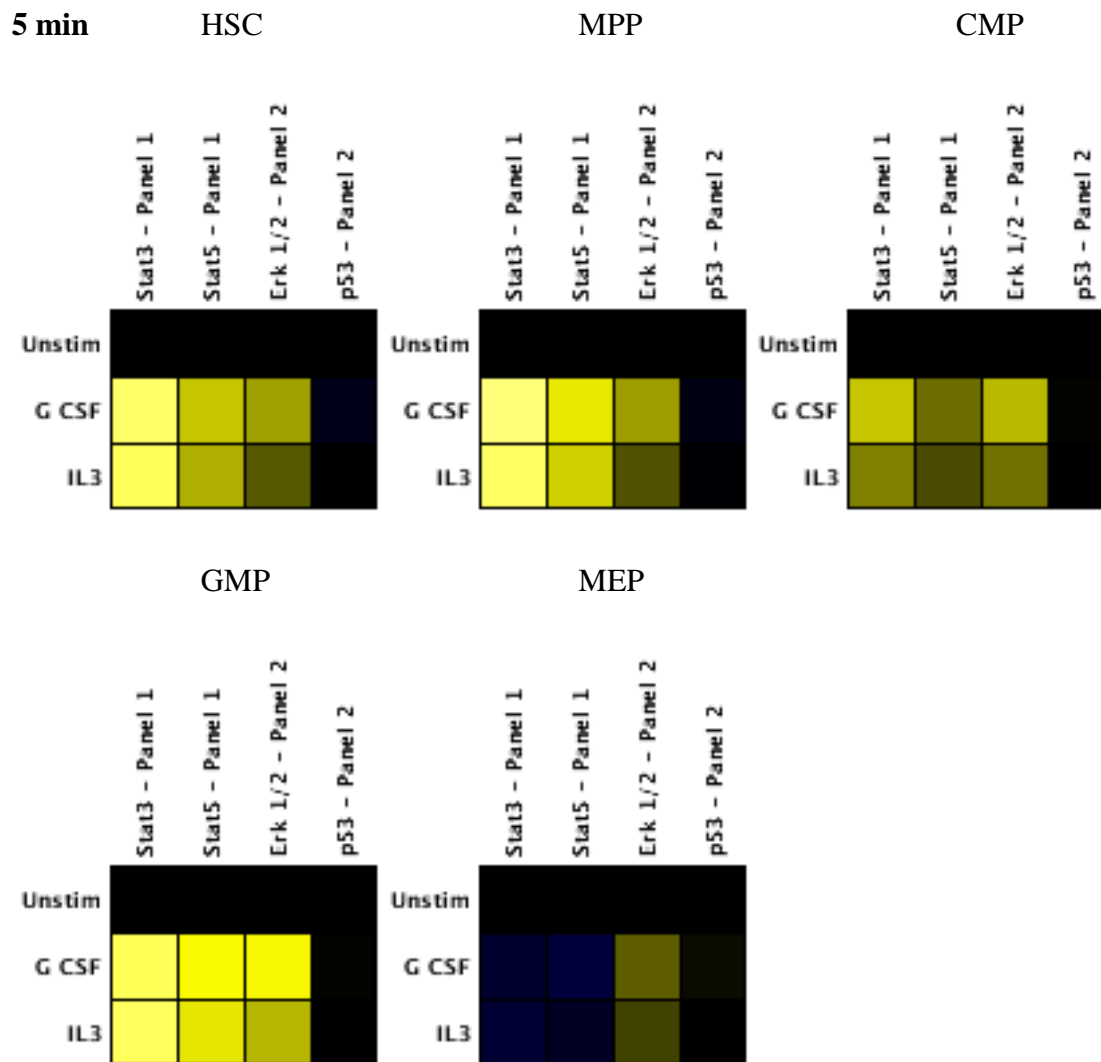
15 min

HSC, MPP, CMP, GMP, MEP
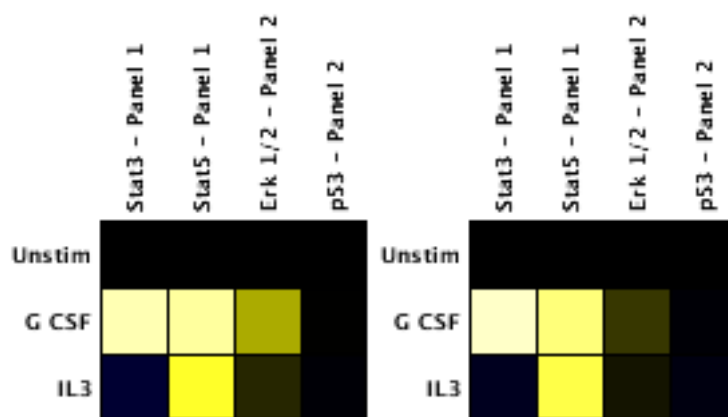
Transformed Ratio of Medians by First Row

−2.93   −1.46   0.00   1.46   2.93
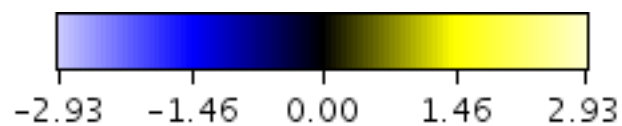
Figure 9. Allogeneic leukapheresis product (LP) – 2 donors. Heatmap of the transformed medians by first row of cells responding in each stem and progenitor sub population presented for LP Donor 1:

GMP

MEP
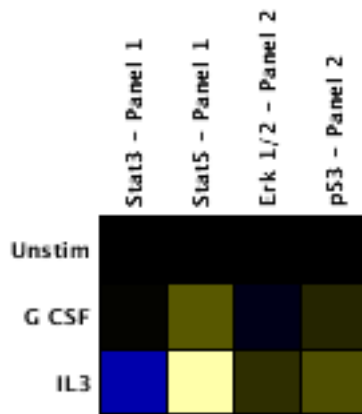
LP Donor 2:

5 min   HSC   MPP   CMP
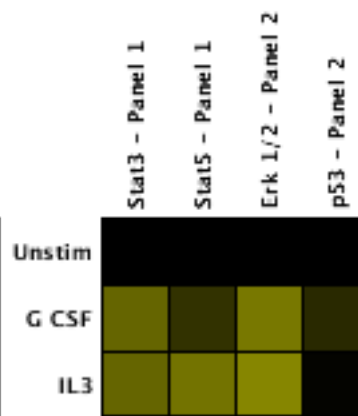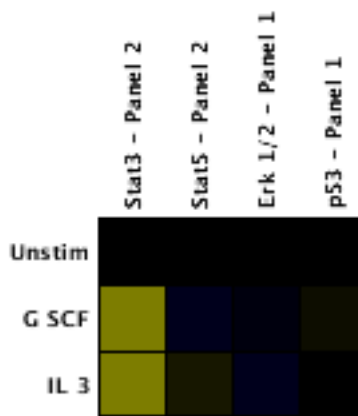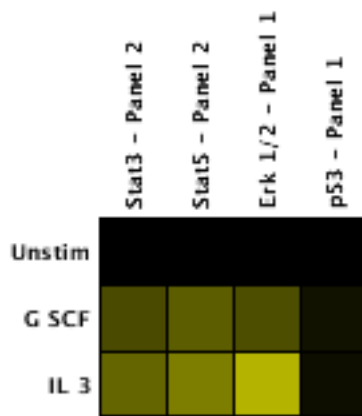
GMP   MEP
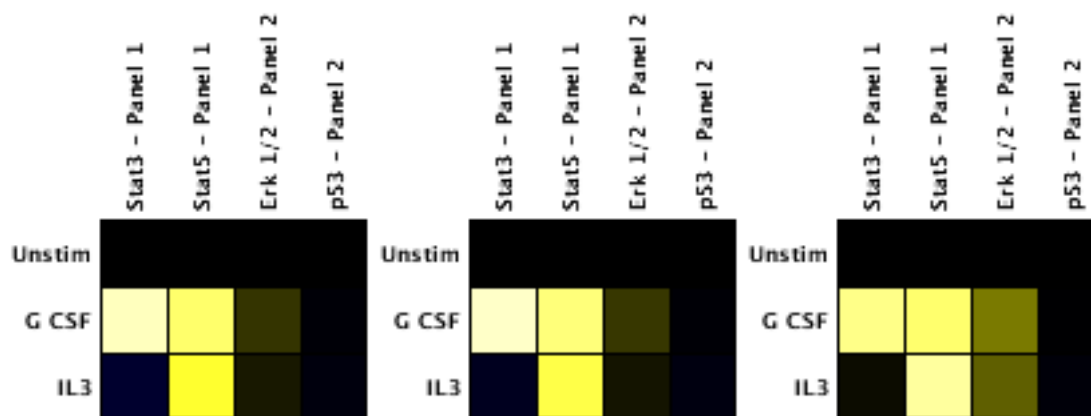
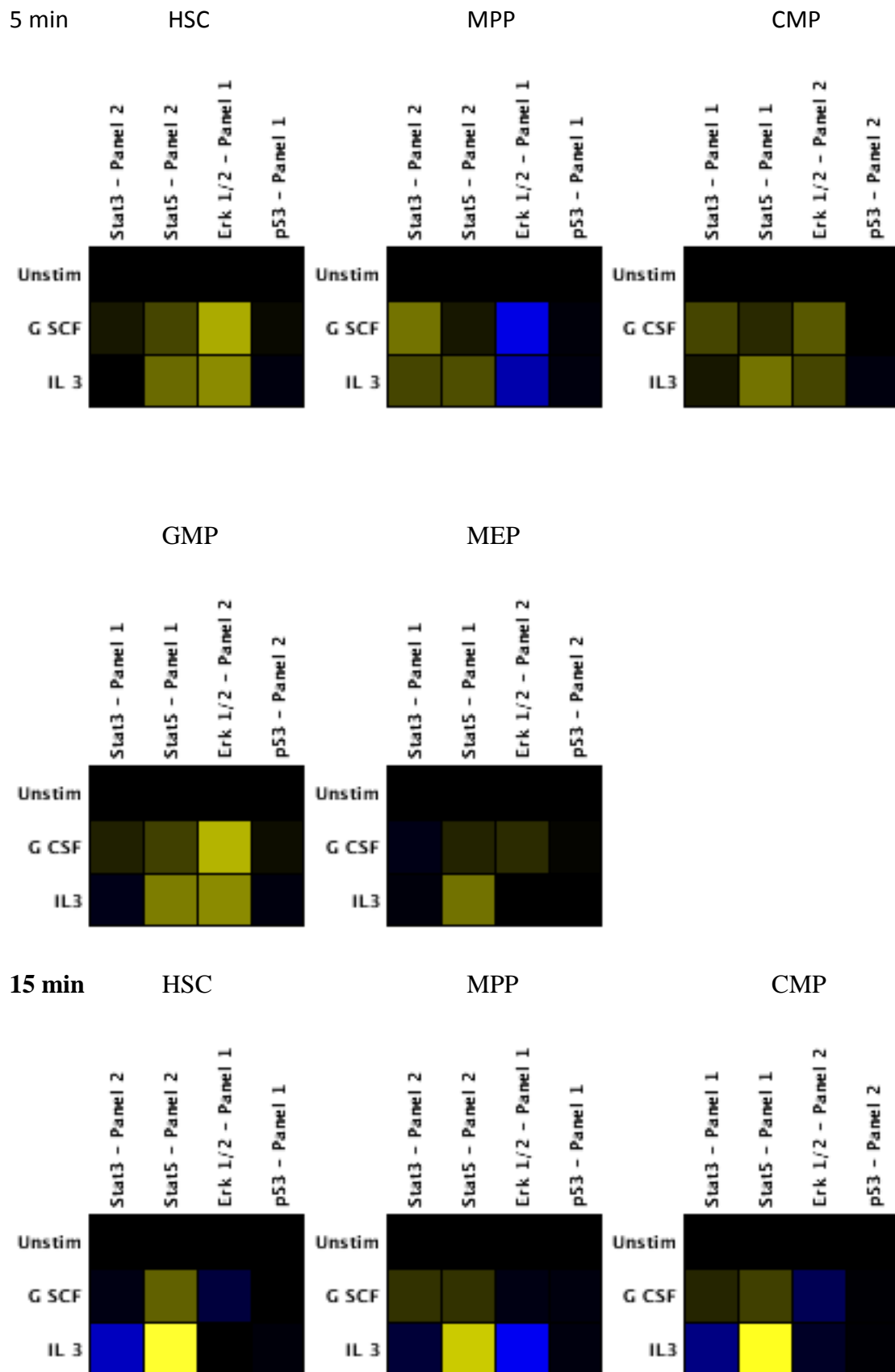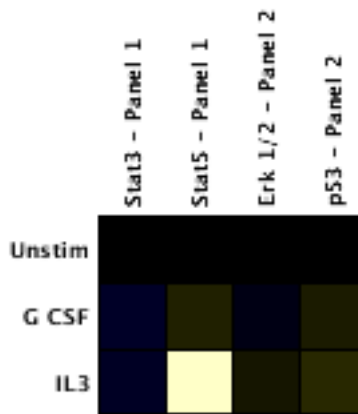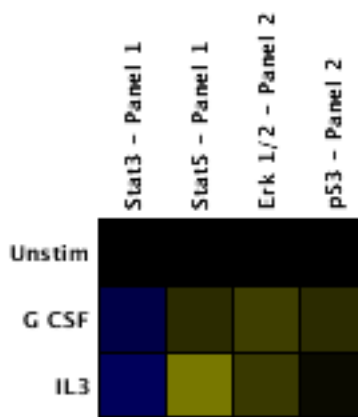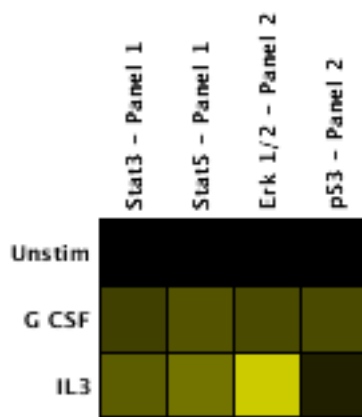**15 min**   HSC   MPP   CMP
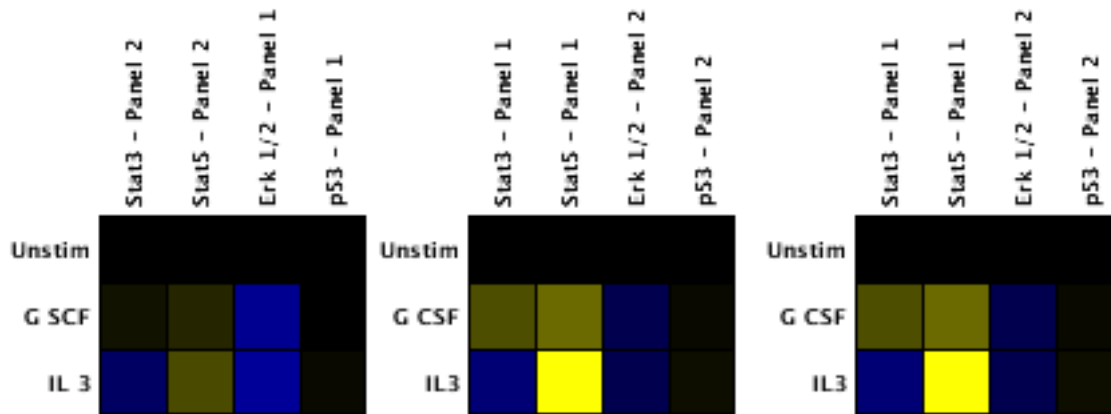
   GMP   MEP
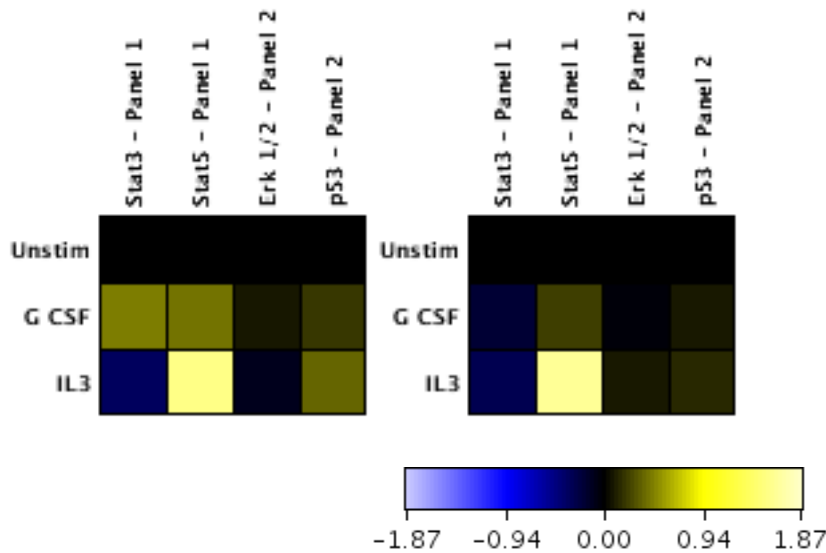
All in all the heatmaps of the two leukapheresis products demonstrates a characteristic lower response to the cytokines G-CSF and IL- 3 than the bone marrow samples. However, in the 15 minutes samples we observe an inhibited response for Stat3 with IL-3 stimuli opposite to Stat5(tyr694) and IL-3 were we could observe an increased response after 15 minutes in the GMP and MEP populations than for the other three less differentiated populations. The Erk1/2 and p53 signals are low or inhibited in response to both cytokines.

4.2.2 Signaling patterns for Stat3 of unstimulated sample from bone marrow and leukapheresis product.

The Stat3(tyr705) histogram of the non-stimulated sample from the leukapheresis product revealed a bimodal peak we could not observe for the non-stimulated bone marrow samples for the different sub populations. This is likely because the cells have a much higher basal phosphorylation level compared to the bone marrow cells. Taken together it is possible that these results demonstrate that G-CSF stimulated sub populations represent biochemically distinct subsets. Bimodal peaks can indicate that a particular population contains sub-populations of cells that need to be identified with more surface markers. Furthermore it is an indication of an already phosphorylated state due to the G-CSF treatment of the donors.

Figure10. Comparison of normal bone marrow phospho-signaling profile for Stat3(tyr705) and allogeneic leukapheresis product.

Tube Name: Unstim 5 min Stat 3/5

Population	#Events	%Parent	STAT3 PE-A Median	STAT5 Alexa fl... Median
CMP	1,594	47.1	457	287
GMP	705	20.8	574	351
MEP	736	21.7	135	270
HSC	627	14.1	479	273
MPP	2,405	54.1	241	301

Tube Name: G CSF 5 min Stat 3/5

Population	#Events	%Parent	STAT3 PE-A Median	STAT5 Alexa... Median
CMP	914	43.2	469	342
GMP	472	22.3	617	408
MEP	542	25.6	120	311
HSC	858	18.3	457	308
MPP	2,042	43.5	291	330

Tube Name: Unstim Stat 3_5

Population	#Events	%Parent	Stat3 PE-A Median	Stat5 Alexa... Median
CMP	2,900	28.8	164	401
GMP	4,390	43.6	179	450
MEP	1,003	10.0	125	364
HSC	346	27.2	167	370
MPP	546	42.9	149	330

Figure10. The figure demonstrates the bimodal peak in MPP, CMP and GMP compartment in the unstimulated sample of the allogeneic LP product. In the HSC and MEP compartment it is not observed a bimodal peak. Bone marrow samples do not demonstrate this pattern for any of the compartments in the non-stimulated samples.

4.3 Evaluation of the antibody panel for surface markers evaluated both in vivo and in vitro.

4.3.1 Flow Cytometry Activated Cell sorting (FACS)

To be able to evaluate the functionality of the different stem- and progenitor popluations and the gating strategy in the flow cytometry analysis part, a Flow Cytometry Activated Cell Sorting (FACS) protocol was established. The gating strategy for the surface markers were approximately the same as for the intracellular phospho-flow protocol as demonstrated in figure 7a. A postsort analysis was performed on the sorted populations as shown in figure 7a. To be able to keep the cells alive it was not possible to include the intracellular antibodies. Validation of signaling in sorted populations was not performed, but should be considered for future experiments to internally confirm signaling responses in the defined cell populations.

Figure 11a. FACS viable cell sorting of HSC, MPP, CMP and GMP sub populations of CD34 positive cells, 50 000 events were recorded while sorting.
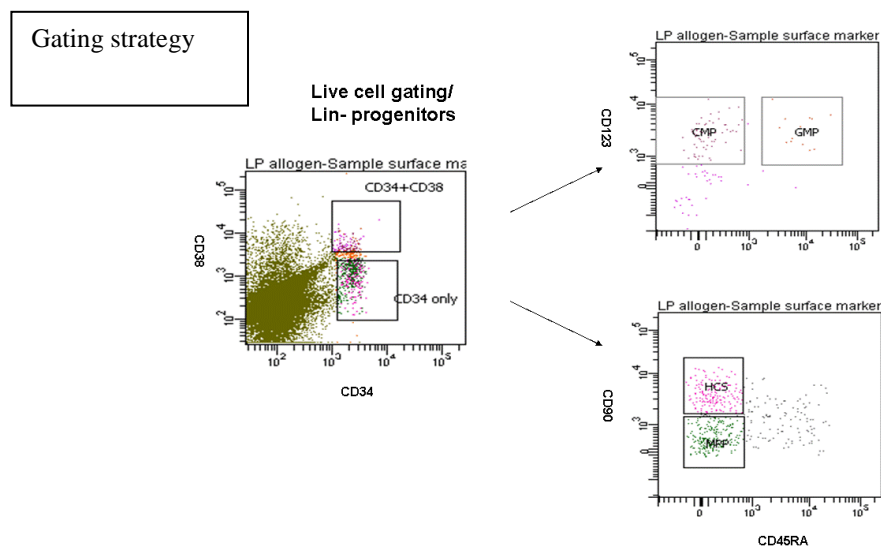
Post sort analysis of HSC, MPP, CMP and GMP
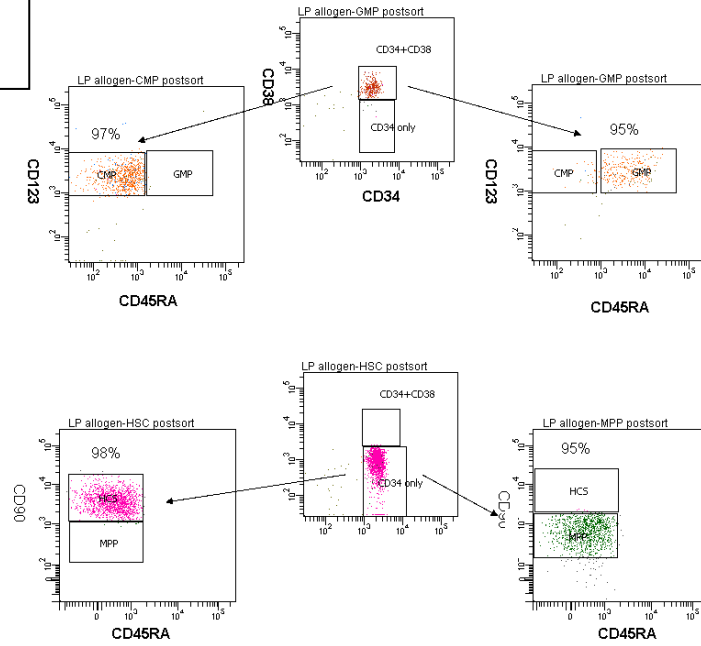
Figure 11b: Analysis of rest-sample day after sorting
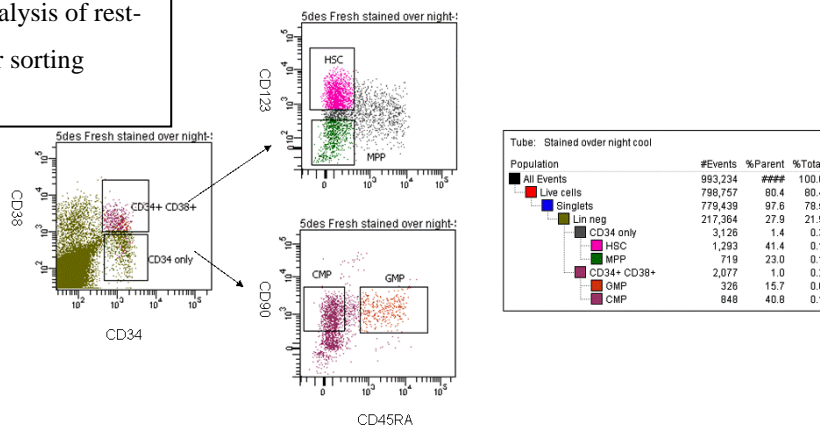
Figure 11b show that the sample can be stored over-night in a cold room at approximately +6°C before sorting. Both the viability and the antibody pattern remain unchanged from the day before.

Next day viability check on the sorted populations after 11 hours in cooling room showed the same result as the day before, viability (according to light scatter plot) at 96 % and the population was falling into the same purity gate. Figure not shown.

4.3.2 In vitro evaluation of sub populations of CD34+ hematopoietic cells.

Colony Forming Unit (CFU) assay was performed twice with FACS sorted sub populations (HSC, MPP, CMP, GMP) in duplicates in a 24 well plate. The first time the colonies were characterized and photographed but not classified as colonies, see figure 8. The HSC growth pattern showed small dense white colonies, some larger single white cells and some red small

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colonies, 46 colonies in total. MPP colonies were characterized by both small colonies and small and large cells in colonies together, and some small red cell colonies, 47 in total. CMP had both small and large cells in colonies and some colonies with only big white cells and few red cell colonies, 35 in total. GMP had 7 colonies in total, large cells in colonies with small cells among them. The second time the different colonies were counted after 14 days for each sub population. Colony counting doublet average:

Table 3.

	CFU-G	CFU-M	CFU-GM	BFU-E
HSC	21	27	36	38
MPP	78	58	34	56
CMP	>100	39	>100	>100
GMP	8	22	25	9

Cultured cells were: 50 000 (HSC), 30 000 (MPP), 30 000 (CMP) and 10 000 (GMP)

Figure 12. CFU assay of CD34+ sub populations, HSC, MPP, CMP and GMP.
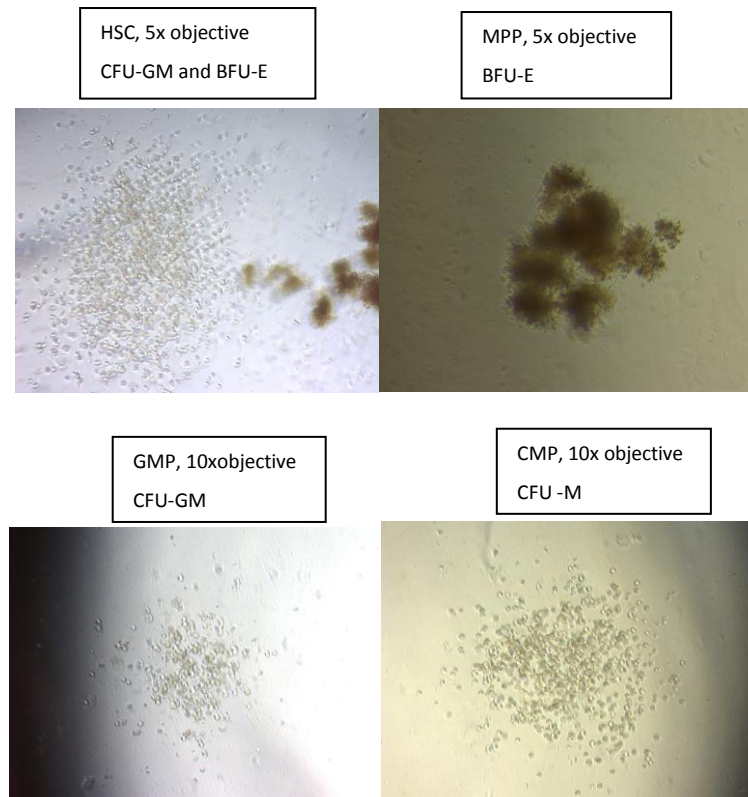
4.3.3 Engraftment and localization of human hematopoiesis of NSGS mice after transplantation of human sub populations of CD34+ cells.

Sorted HSC, MPP, CMP and MEP subpopulation from two donors were injected into NSGS[72] mice for detection of human repopulating cell activity. The results obtained so far by flow cytometry for human CD45 and CD33 show no positive events. Leukapheresis products have been FACS sorted and the four sub populations HSC, MPP, CMP and GMP have been transplanted twice. A not transplanted NSGS mouse is kept as a negative control. Samples from peripheral blood from both the transplanted animals and the negative control mouse have been analyzed every second week since week six after transplantation and it will continue until week 20 were the mice will be sacrificed and samples taken for flow cytometry analysis from bone marrow, spleen and peripheral blood to assess long term human engraftment.

Figure 13a. Flowcytometric analysis of NSGS mice peripheral blood stained with human CD45 and human CD33 to detect leukocytes and cells from the myeloid compartment. Group 1 (HSC, CMP, GMP) analyzed 10 weeks after transplantation. MPP-mouse was sacrificed after four weeks due to an unspecified tumor in relation to the scapular bone.
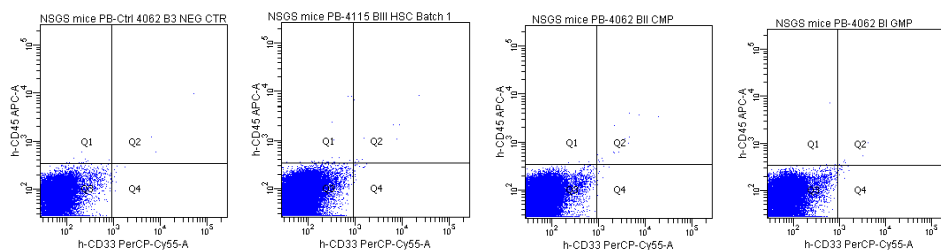
Figure 13b. Flowcytometric analysis of NSGS mice peripheral blood stained with human CD45 and human CD33 to detect leukocytes and cells from the myeloid compartment. Group 2 analyzed 6 weeks after transplantation.
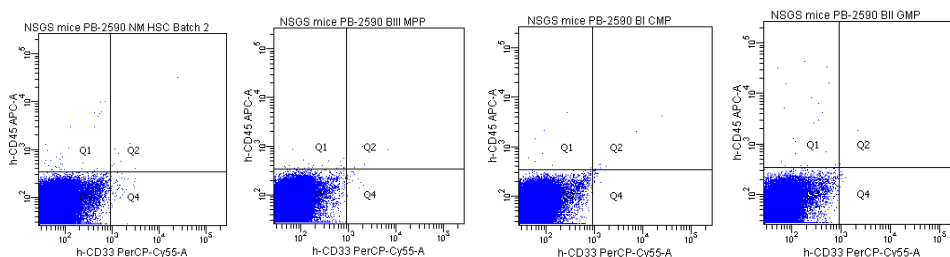
In neither groups there are significant human CD45 positive events at 6 weeks and the study continue until week 20 after transplantation.

5. Discussion

This thesis contains of three parts. The first part describe establishment of a robust antibody panel and protocol for staining of hematopoietic stem- and progenitor cells to study intracellular signal transduction. The material examined was bone marrow donated from healthy individuals, leukapheresis product from healthy donors for allogeneic bone marrow transplantation. A separate protocol for cord blood has not been done due to logistics problems. Five subpopulations of CD34+ cells from healthy individuals have been defined by a set of antibodies (Lin-, PARP, CD34, CD38, CD90, CD45RA,CD123) and examined for the phosphorylation level in Stat3(tyr705), Stat5(tyr694), Erk 1/2. In addition the total level of the cell stress sensor and tumor suppressor protein p53 was examined. In the second part a FACS sorting protocol using the same surface markers was established for leukapheresis product with the purpose of isolating the different subpopulations. In the third part, cells from the sorted and isolated cells from part two were evaluated for protocol and the gating strategy using the Colony Forming Unit *ex vivo* method, and also xenotransplanted into NSGS mice.

Multicolour flow cytometry is the main technique employed for analysis of intracellular signalling in this thesis. Other methods to study signalling proteins include gene sequencing, immunohistochemistry, mass spectrometry, western blot and micro array analysis. The advantage of measuring protein levels and level of protein modification by flow cytometry is the ability to determine heterogeneity within specific subpopulations of cells. Possible limitations and weaknesses of flow cytometry will be discussed.

5.1 Establishment of antibody panel and protocol for staining

5.1.1. Sample handling and preparation

Proper sample handling is essential to avoid induction of artefacts. Bone marrow samples from healthy individuals have been frozen for different periods of time and some of samples were leftover product after removing of B-cells. Since the bone marrow samples are limited material it was not possible to control the sampling, storage or freezing procedures. Handling procedures may affect the final results regarding antigen preservation, cell viability and activation status. Given that total p53 is increased in cells exposed to diverse stress signals like DNA damage, ribosomal stress and hypoxia [73] it

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was not expected to observe any increase in p53 signal for the healthy bone marrow and allogeneic leukapheresis products. However, we find a slight p53 increase both for untreated cells and stimulated cells in the bone marrow samples in figure 7. The response of total p53 for the bone marrow samples are more consistent compared to the leukapheresis products. This may indicate that the quality of fresh samples handled within two hours after end of collection is more ideal for signalling studies than frozen material. These data propose that total p53 can be used as an indicator of sample quality combined with a marker for cell death and/or apoptosis and a gating strategy that include exclusion of debris and non-typical events in a light scatter plot.

5.1.2 Antibodies, cytokine concentrations and time points

Antibodies recognize antigenic determinants, also called epitopes on their antigen. The specificity to epitopes and sensitivity of antibodies varies a lot from clone to clone. In this thesis the antibodies of choice are parts of an established panel used in several papers.[23, 74] The panel in this study is built after a model from Gibbs paper and all potential antibodies against each epitope are not tested. The results from the establishment of the panel showed that the CD90 (5E10) expression was highly increased after permeabilization when the surface staining was done together with the intracellular staining. As a consequence CD90 staining was done before permeabilization. Another fluorochrome with the same antibody clone was tested with the same result. No further investigation was done to examine the phenomenon. Also to assess four signaling proteins (total p53, phosphorylation of Stat3, Stat5, and Erk1/2) in one panel with the choice of fluorochromes in the thesis without decreasing the sensitivity of the antibodies was not possible and the conclusion was to use two panels, one for Stat3 and Stat5 and one for p53 and Erk1/2 with the identical surfacemarker antibodies for both tubes.

The concentration of cytokines in the thesis is also chosen on the basis of the paper by Gibbs et al and the time points were chosen according to earlier work done by the Gjertsen lab [75]. To be able to determine the optimal concentration of the cytokines and time points for detection of optimal phospho-signalling, a concentration titration and a time point study should be performed.

5.1.3 Controls

Controls included in the study are quality controls for the flow cytometer (CS&T), unstained control, compensation controls, gating controls/biological controls. A matched isotype control for p53 antibody was tested but no unspecific staining was found due to the isotype control. The isotype control and the unstained control had identical levels. The conclusion was not to include the isotype control in the panel.

Isotype controls are meant to account for unspesific staining but not for fluorochrome spillover. There are two important considerations to take into account with using isotype controls. The first limitation is that individual antibody conjugates have various levels of background staining, depending on specificity, concentration and fluorochrome:antibody ratio (F/P ratio) among other variables. The second limitation is that it does not account for fluorochrome spillover from other detectors. This can be overcome by including all relevant antibodies in the panel along with the isotype control in the detector of interest and you will have a Fluorescence Minus One (FMO) control.

For the surface marker CD38 in the panel where there is no clear division between positive and negative populations a gating control (FMO) was included. The gating control can be a biological comparison control (unstimulated or irrelevantly stimulated cells) or an isotype control/FMO control. For the signalling antibodies the unstimulated samples provided the best control to distinguish positive events from negative events [71].

5.2 Signal transduction assay

Part of the objective of this thesis was to develop a robust protocol to detect activated signalling pathways within the five defined subpopulations of CD34+ cells in the samples by simultaneously staining for surface markers and intracellular phosphorylated proteins for flow cytometry. By using a flow cytometric strategy fewer cells are required when compared to alternative techniques including western blotting. Western blotting and flow cytometry has previously been compared and provide similar qualitative results for phosphorylated protein analysis [70]

Flow cytometry however allows one to identify individual cells with different responsiveness, rather than a mean value for the total cell population without

separating cells prior to stimulation and analysis. For the five subpopulations investigated in this thesis it is in practice impossible to use western blot because of the small amounts of cells located in each sub population.

5.2.1 Permeabilization step is critical for the surface epitopes

Several protocols for detection of intracellular/nuclear epitopes is described [69, 70] and fixation and permeabilization steps are widely discussed. In this study permeabilization conditions were tested for concentration of methanol and incubation temperature. The permeabilization step is crucial to allow passage of the intracellular antibodies without destroying the structural integrity of the cells. Based on this both 90% methanol and 70% methanol was tested in combination with incubation temperature ranging from minus 20°C, on ice until or up to +4°C in the fridge. Incubation time was also tested, 10 minutes, 15 minutes and 30 minutes. We observed a decrease in the intensity of CD38 signal and a total positive signal of CD90 when 90% methanol was used and the cells were kept on ice or in -20. The phosphorylated epitopes was still intact when cells were exposed to 70% methanol for 30 minutes in the fridge and the cell surface labelling was sufficient.

5.2.2 Comparison of signalling patterns from bone marrow and leukapheresis product

Based on the observations of the signalling patterns after cytokine exposure the most interesting findings for us were a significant difference in the signalling pattern between specimens. While conclusions from studies using model systems have described the HSC sub population only responding to a set of early acting cytokines such as SCF and TPO [76, 77]. This study demonstrates a heterogeneous and substantial response for the two timepoints to single stimuli based on the mean fluorescence intensity ratio on unstimulated samples compared to stimulated samples and the results are confirmed by the study of Gibbs et al[23]. It was not possible in this study to observe the decreasing response from G-CSF in the more mature progenitor populations. On the other hand we observed a similar response to IL 3 from the Stat3(tyr705) and Stat5(tyr694) antibody. The bone marrow samples have an increased response from Stat5 from all the five sub populations as for the study done by Gibbs et al. An important observation from the two leukapheresis samples is a negative expression for the 15 minutes activation samples.

This is a very interesting observation and to further investigate this more donors are needed to make the statistics more robust. In addition confirmation of the result from other methods like proliferation in direct response to G-CSF and/or IL-3, receptor status with antibody staining for G-CSF receptor (CD114) and IL-3 receptor (CD123). CD123 is actually included in this study as a marker for progenitors. To conclude on the different responses from the frozen bone marrow samples from five donors and fresh leukapheresis products from two donors the most central observations for this study are the importance of viability of samples and that optimal time of stimulation vary between cytokines. This demonstrates that the context in which responses are measured could have a considerable influence on the signals observed.

In the leukapheresis products, our findings of a bimodal peak in the unstimulated sample and an approximately identical pattern in the G-CSF stimulated samples are very interesting, figure 10. Different Stat3 phosphorylation levels were demonstrated for the five different populations. These patterns were only found in the leukapheresis product, and not present in the bone marrow samples. The donors were treated with G-CSF before the collection of leukapheresis product and it is likely that our signalling analysis is reflecting this stimulation in vivo. The 1.5 hour resting period before activation with cytokines seems not enough to reset the cells to zero activation. This observation should be further investigated.

In summary, we provide a protocol that allows successful discrimination of several CD34+ sub populations in bone marrow and leukapheresis products. This permits detection of intracellular signal transduction.

It should be reminded that the aim of this study was not to determine differences of signal transduction within cell subpopulations among healthy donors. In fact, the goal was to assess whether the established protocol can be used as a tool to study expression of signal transduction in different material like bone marrow, leukapheresis products and cord blood. The study does not fulfill the aim of establish the protocol for cord blood and the project will therefore be continued.

5.3 Evaluation of the sub populations by colony forming unit assay.

FACS sorting of four out of five sub populations (HSC, MPP, CMP and GMP) was done prior to the experiments and a control of the purity of the four different populations.

Colony forming unit assay were used to confirm the ability for the four populations to form colonies. After adjusting the amount of cells needed performing this assay, the results showed an increased amount of colonies in each sub population. The medium used; MethoCult cat. No. 4433 is not the commonly used for this type of set up, but according to the manufacturer it will give the same result as the cat. No. 4434 Classic. The medium used contained phytohemagglutinin leukocyte conditioned medium (PHA-LCM) as a source of colony-stimulating factors, plus recombinant human erythropoietin (EPO). The findings suggest that the antibodies used and the gating strategies when the cells were sorted are well defined. Furthermore, the colonies performed from the four populations showed a difference in morphology that further indicate different proliferation and/or differentiation steps for the sub populations represent are expressed through the result of the assay.

5.4 Evaluation of the sub populations by xenotransplantation with NSGS mice.

The NSGS mice strain is developed by cross breeding the NSG strain with the NSS (NS mice transgenic for the human cytokines SCF, GM-CSF and IL-3) strain [72]. This strain promotes increased human myelopoiesis compared to the NSG mouse. The NSGS strain have not previously been used in-house for xenografts with healthy hematopoietic stem and progenitor cells so it is difficult to estimate the time point for when to expect the transplanted human cells to proliferate and differentiate to a measureable level in peripheral blood. It has been reported engraftment after 6 weeks from patient cells affected with Myelodysplastic syndrome [78]. However, we have not yet observed any positive events in the mice PB samples but the experiment are still going and samples are taken every two weeks and stained for human CD45 and human CD33. The project will continue until week 20 after xenograft, then the animals will be sacrificed and samples taken for flow cytometry analysis from bone marrow, spleen and peripheral blood to assess long term human engraftment. In theory this model should be ideal for this study. However, it can be discussed if this is a good model for evaluating the different sub-populations as long as we have not seen any reports on effect of healthy hematopoietic stem- and progenitor cell xenografts with this mouse strain. On the other

hand, it is a very interesting experiment if we will be able to detect human blood cells for future projects.

The work done here provides a small glimpse into the complex network of signalling pathways that regulate growth, differentiation and function of human hematopoietic cells. Despite the enormous amount of information that is reported in recent years regarding signalling networks many challenges remain. Among others to understand how multiple signalling networks act together to create a given biological fate.

5.5. Future perspectives

Obtained results in this thesis certainly provide an important tool for further studies of signalling pathways of human hematopoietic stem- and progenitor cells. Xenograft assays are still going and another project are using the antibody panel for studying xenografts with patient material from an acute myeloid leukemia. To further refine the antibody panel the goal is to combine all four signalling markers in one panel. This will be a benefit considering the great cell loss due to the washing steps in the protocol. The continuous project will also include cord blood. So the next step is to establish a protocol also for this type of material. Furthermore, it will be interesting to compare frozen and fresh material and study the signalling effects. To study the interesting findings of the bimodal peaks in the unstimulated samples of the leukapheresis products we need to study more samples and evaluate the surface markers.

It will be of interest to establish an antibody panel were the aim is to study cytokine receptor levels, in particular sort cells based on different expression of the G-CSF receptor (CD114). By repeating the xenograft experiment with sub populations with high and low expression of G-CSF receptors we may validate the study of Gibbs and co-workers [23] were they found that long term human HSC activity is enriched in the G CSF [neg/low] population. For the ongoing project it is also to interesting to include fresh samples of autologous leukapheresis donors and compare the results to the allogeneic donors.

Protein phosphorylation is one of the most biologically significant post translational modifications in cells, and requires sophisticated methods for detection. The application

of genomic tools like quantitative mass spectrometry (SILAC) to study post-transcriptional protein modification may be an important supplement in studying phosphorylation of proteins and will give valuable insight in the signal transduction processes.

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